Aus dem Institut für Tierzucht und Vererbungsforschung der
Tierärztlichen Hochschule Hannover

Molecular genetic analysis of congenital deafness in Dalmatian dogs

INAUGURAL-DISSERTATION
zur Erlangung des Grades einer
DOKTORIN DER VETERINÄRMEDIZIN
(Dr. med. vet.)
durch die Tierärztliche Hochschule Hannover

Vorgelegt von
Simone Gerlinde Rak
aus Kehl

Hannover 2003

Scientific supervisor: Univ.-Prof. Dr. Dr. O. Distl

Examiner: Univ.-Prof. Dr. Dr. O. Distl
Co-examiner: Univ.-Prof. Dr. H.-J. Hedrich

Oral examination: 24.11.2003

This work was supported by a grant from the Gesellschaft zur Förderung Kynologischer Forschung (GKF) e.V., Bonn, Germany.

Dedicated to my family
and to Markus

Parts of this work have been published in the following journals:

1. Animal Genetics 33 (2002) 389-90
2. Chromosome Research 10 (2002) 407-10
3. Cytogenetic and Genome Research 101 (2003) 130-35
4. Cytogenetic and Genome Research 97 (2002) 140A
5. Cytogenetic and Genome Research 97 (2002) 140B

Contents

List of abbreviations

A	adenine
Acc.	accession
AEP	akustisch evozierte Potentiale (acoustically evoked potentials)
AT	annealing temperature
BAC	bacterial artificial chromosome
BAER	brain stem auditory evoked response
BLAST	Basic Local Alignment Search Tool
bp	base pair
C	cytosine
CCD	charged coupled device
CD3E	CD3E antigen, epsilon polypeptide (TiT3 complex)
CDH23	cadherin related 23
cDNA	copy desoxyribonucleic acid
CFA	chromosome of *Canis familiaris*
CLDN14	claudin 14
cM	centiMorgan
COCH	coagulation factor C homolog, cochlin
COL11A2	collagen, type XI, alpha 2
CSD	congenital sensorineural deafness
DAPI	4',6'-diaminidino-2-phenylindole
DEAC	diethylaminomethylcoumarin
DFN	X-linked deafness locus
DFNA	autosomal dominant deafness locus
DFNA5	deafness, autosomal dominant 5
DFNB	autosomal recessive deafness locus
DIAPH1	diaphanous homolog 1 (Drosophila)
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid

dNTPs	deoxy nucleoside 5'triphosphates (N is A,C,G or T)
dUTP	deoxy uracil triphosphate
E. coli	*Escherichia coli*
ECL	enzyme chemiluminescence
EDN3	endothelin 3
EDNRB	endothelin receptor type B
EDTA	ethylenediamine tetraaceticacid
EMBL	European Molecular Biology Laboratory
EST	expressed sequence tag
EYA4	eyes absent homolog 4 (Drosophila)
F	forward
FISH	fluorescence *in situ* hybridisation
G	guanine
GJA1	gap junction protein, alpha 1, 43kD (connexin 43)
GJB2	gap junction protein, beta 2, 26k (connexin 26)
GJB3	gap junction protein, beta 3, 31kDa (connexin 31)
GJB6	gap junction protein, beta 6 (connexin 30)
GKF	Gesellschaft zur Förderung Kynologischer Forschung (Society for the Advancement of Cynological Research)
GLUT4	glucose transporter 4
GTG	giemsa trypsin giemsa
h_E	expected heterozygosity value
h_O	observed heterozygosity value
HSA	chromosome of *Homo sapiens*
IBD	identical by descent
IMAGE	integrated molecular analysis of genomes and their expression
IRD	infrared dye
KCNQ4	potassium voltage-gated channel, KQT-like subfamily, member 4
kb	kilobase
LINE	long interspersed nuclear element
LOD	logarithm of the odds

M	molar
Merlin	multipoint engine for rapid likelihood inference
MITF	microphthalmia-associated transcription factor
MS	microsatellite
MYH9	myosin, heavy polypeptide 9, non-muscle
MYO3A	myosin IIIA
MYO6	myosin VI
MYO7A	myosin VIIA
MYO15A	myosin XVA
NCBI	National Center for Biotechnology Information
NPL	nonparametric linkage
OMIM	Online Mendelian Inheritance in Man
OTOF	otoferlin
p	probability
PAX3	paired box gene 3 (Waardenburg syndrome 1)
PCR	polymerase chain reaction
PIC	polymorphism information content
POU3F4	POU domain, class 3, transcription factor 4
POU4F3	POU domain, class 4, transcription factor 3
QTL	quantitative trait locus
R	reverse
RH	radiation-hybrid
RPCI	Roswell Park Cancer Institute
RZPD	Resource Center/Primary Database, Berlin
SAS	Statistical Analysis System
sd	standard deviation
SINE	long interspersed nuclear element
SLC26A4	solute carrier family 26, member 4
SOX10	SRY (sex determining region Y)-box 10
SSC	sodiumchloride-sodiumcitrate
STRC	stereocilin

STS	sequence-tagged site
T	thymine
TECTA	tectorin alpha
THY1	Thy-1 cell surface antigen
TMC1	transmembrane, cochlear expressed, 1
TMPRSS3	transmembrane protease, serine 3
U	unit
USH	Usher syndrome
UTR	untranslated region
WFS1	Wolfram syndrome 1 (wolframin)
WS	Waardenburg syndrome

Chapter 1

Introduction

Introduction

Introduction

Congenital sensorineural deafness (CSD) has been reported in a variety of mammal species, ranging from humans to mice, dogs, guinea pigs, and mink. Among breeds of dog, none suffers from this inherited affliction with greater frequency than the Dalmatian. Inherited deafness has been recognised in this breed for nearly a century. Although several studies have demonstrated that deafness in Dalmatians is an inherited disease, no universally accepted mode of inheritance has yet been identified nor has the number of contributing genes or a disease-causing gene.

A segregation analysis of the German Dalmatian dog population showed that a mixed monogenic-polygenic model with a major recessive gene and eye colour as covariate best explained the segregation of affected animals in the pedigrees. As breeding with blue-eyed Dalmatian dogs and unilaterally or bilaterally deaf dams and sires is not permitted according to the German animal protection law, the hearing status of these animals has to be tested using the brain stem auditory evoked response (BAER) test. However, it has been shown in recent years that auditory testing does not seem to be an effective way of clearly reducing the high incidence of deafness in this breed. Because deaf dogs are difficult to handle, most puppies suffering from bilateral hearing loss are euthanised. Therefore it seems to be a matter of urgent necessity to characterise the genes responsible for CSD in Dalmatians in order to develop a molecular genetic test for the identification of genetic carriers.

Over the past decade it has become increasingly clear how far structural and functional homologies at the gene level extend across even distantly related species. Thus, the number of deafness-causing gene mutations already identified in humans and mice makes these organisms appear to be suitable models for the molecular genetic approach to canine deafness. The purpose of this thesis is to select suitable human or murine candidate genes for CSD in Dalmatians, to map them to the canine genome, and to develop a candidate gene-associated marker set. This trait-specific set of markers is intended for linkage analysis with CSD in an affected-pedigree-member design for German Dalmatian dogs. The detection of significant linkage between candidate gene-associated markers and CSD would indicate that it is highly probable that the respective genes are involved in the development of the disease. The present

study is intended to identify these canine genes out of a large set of deafness-causing candidate genes in order to clarify the molecular genetic basis of CSD in Dalmatian dogs.

Overview of the chapter contents

Chapter 2 reviews the findings concerning CSD in different dog breeds, including its prevalence, histopathology, inheritance patterns and phenotypic associations. Moreover, parallels to and differences from human deafness are shown, and the advantages of comparative genomics and different molecular genetic approaches are described.

Chapters 3-7 represent articles that have already been published in different peer-reviewed international journals. These chapters describe the selection criteria, the clone isolation, and the partial sequencing and mapping of in total 24 candidate genes for congenital sensorineural deafness in Dalmatian dogs.

Chapter 8 deals with the development of a candidate gene-associated set of markers. Furthermore, this chapter treats the linkage analysis between the microsatellite-based markers developed here and CSD using affected pedigree members of the German Dalmatian dog population.

Chapter 9 provides a general discussion and conclusions referring to Chapters 1-8.

Chapter 10 is a concise English summary of this thesis, while Chapter 11 is an expanded, detailed German summary which takes into consideration the overall research context.

Chapter 2

Congenital sensorineural deafness in dogs: a molecular genetic approach toward unravelling the responsible genes

Simone G. Rak and Ottmar Distl

Congenital sensorineural deafness in dogs: a molecular genetic approach toward unravelling the responsible genes

Abstract

Deafness is a disorder often diagnosed in different dog breeds; it has been identified as a significant problem for breeders, owners and clinicians. The aetiology can be inherited or acquired, and it must be distinguished between sensorineural and conductive forms of deafness. This paper provides a brief overview of the varieties of findings in different dog breeds and in one particular breed including prevalence, phenotypic and gender associations, histology, modes of inheritance and the number of contributing genes in congenital sensorineural deafness. Moreover, we also point out the parallels and differences in canine and human deafness. We describe molecular genetic approaches to canine hearing loss and discuss how comparative genomics could help reduce the incidence of deafness in the affected breeds and lead to new insights into the molecular mechanism of auditory function for both dog and man, as well.

Introduction

The prevalences of congenital sensorineural deafness (CSD) in dogs has increased in recent decades, primarily as a result of heightened awareness of the disorder among dog breeders, owners and clinicians. Animals with hearing impairment are hardly suitable as working dogs, they have a higher risk of becoming victims of traffic accidents, often seem to be easily startled and therefore have an increased tendency to bite. A deaf puppy requires specialised training by a patient and knowledgeable person and has to be kept on a leash most of the time. As it is often extremely difficult for breeders to find such responsible owners, most puppies with bilateral deafness are euthanised.

In the present study we give a short overview of the aetiology, prevalence, phenotypic and gender associations, mode of inheritance and histological pattern of CSD in different dog breeds and discuss a molecular genetic approach to canine deafness by using comparative genomics.

Sensorineural and conductive deafness

Canine hearing loss can be in inherited or acquired. The most commonly seen forms are (1) inherited congenital sensorineural, (2) acquired later-onset sensorineural and (3) acquired later-onset conductive deafness (Strain, 1996). A more simplified classification distinguishes between conductive and sensorineural deafness (Steffen and Jaggy, 1998). Conductive deafness is the result of a dysfunction caused by problems in the external ear canal or the middle ear space, and it often results in an incomplete hearing loss (Eger and Lindsay, 1997). For example, chronic otitis externa and media with ensuing stenosis and possible occlusion of the external ear canal, impaction from excess cerumen accumulation, rupture of the tympanic membrane, or stiffening or fractures in the bony ossicles can cause this type of deafness. In contrast, sensorineural deafness normally leads to complete uni- or bilateral hearing loss and may be caused by dysfunction of the cochlea of the inner ear, by alterations of the cochlear nerve or of portions of the auditory pathway within the central nervous system. Indeed, it appears that most cases of sensorineural hearing loss in dogs involve only the cochlea and/or the peripheral nerves (Steffen and Jaggy, 1998). However, Ferrara and Halnan (1983) questioned this theory and postulated the central development of deafness in Dalmatian dogs. The causes for sensorineural deafness are diverse, and an inherited alteration of the inner ear structures is only one possibility. Otitis interna, tumours, ototoxic agents (e.g. aminoglycoside antibiotics), trauma and presbycusis can also result in this form of deafness (Strain, 1996).

In cases of suspected sensorineural deafness a conductive form of hearing impairment must first be excluded. Different examinations can be performed, such as otoscopy of the ear canal with an examination of the tympanic membrane, x-ray and computer tomography of the middle ear, or myringotomy to specify the bacteriological/cytological content of the Bullae tympanicae. A reliable method for diagnosis of sensorineural deafness has shown to be the brain stem auditory evoked response (BAER) test, which is a reflection of electrical events within the brain stem as they ascend through the auditory pathway (Sims and Moore, 1984). The BAER test can be carried out either with a bone stimulator, particularly when there is reason to suspect conductive deafness, or with an air-conducted click produced by earphones (Strain *et al*., 1993). It is also possible to diagnose dogs suffering from unilateral hearing impairment with this test.

Prevalence of canine deafness

Canine congenital deafness has often been reported in the literature and occurs in more than 54 different breeds of dogs, according to Strain (1996). However, because of the various possible acquired causes of congenital deafness and in the absence of breeding studies for many breeds, it cannot be confirmed that all of those cases were inherited. The breeds with the highest prevalence include Dalmatian, Bull Terrier, English Cocker Spaniel, English Setter, Australian Cattle Dog (Table 1), Australian Shepherd, West-Highland-White-Terrier, Dobermann and Dogo Argentino.

Table 1. Breed-specific deafness prevalence in dogs (adapted from Strain, 2003a).

Breed	Dogs tested	Unilaterally deaf (%)	Bilaterally deaf (%)	Total deaf (%)
Australian Cattle Dog	296	12.2	2.4	14.6
Bull terrier	665	9.9	1.1	11.0
White	346	18.0	2.0	20.0
Coloured	311	1.3	0.0	1.3
English Setter	3656	6.5	1.4	7.9
English Cocker Spaniel	1136	5.9	1.1	7.0
Parti-coloured	1067	5.9	1.1	7.0
Solid-coloured	60	1.7	0.0	1.7

The incidence of deafness is highest in Dalmatian dogs, of which 16.5 to 29.9% exhibit unilateral or bilateral hearing loss (Famula *et al*., 1996; Holliday *et al*., 1992; Juraschko *et al*., 2003a; Muhle *et al*., 2002; Strain, 2003a; Wood and Lakhani, 1997) (Table 2). In an American study Strain (2003a) demonstrated that the incidence of deafness in other BAER-tested dog breeds appears to be approximately half of that of Dalmatians. The frequency of unilaterally affected animals is generally higher than that of totally deaf animals. Indeed, most of the studies mentioned above show that about two to three times more Dalmatians are unilaterally deaf than bilaterally.

Table 2. Deafness prevalence in Dalmatian dogs.

Authors	Dalmatians tested	Unilaterally deaf (%)	Bilaterally deaf (%)	Total deaf (%)
Strain, 2003a (USA)	5333	21.9	8.0	29.9
Holliday *et al*., 1992 (USA)	900	21.0	7.0	28.0
Famula *et al*., 1996 (USA)	825	-	-	26.0[a]
Juraschko *et al*., 2003a (Germany)	1899	12.3	7.4	19.7
Wood and Lakhani, 1997 (UK)	1234	13.1	5.3	18.4
Muhle *et al*., 2002 (Switzerland)	254	9.4	7.1	16.5
Greibrokk, 1994 (Norway)	1843	-	-	4.9[b]

[a] No differentiation between uni- and bilaterally affected dogs.

[b] Results obtained without BAER testing.

Pigmentation and gender associations

Numerous investigators have looked for phenotypic associations of other traits with deafness. The association of CSD with pigmentation pattern in dogs has been described in published reports for more than a century (Rawitz, 1896). In breeds with white and non-white phenotypes (e.g. Bull Terrier, English Cocker Spaniel), there is a clearly increased prevalence in the white phenotype (Strain, 1999, 2003a). The most commonly observed association is between blue eyes (heterochromia iridis) and deafness (Famula et al., 2000; Greibrokk, 1994, Holliday *et al*., 1992; Juraschko *et al.*, 2003a, 2003b; Mair, 1976; Strain *et al.*, 1992; Strain, 2003a). Another characteristic that has been shown to be associated with deafness in Dalmatians is the presence or absence of a patch, a visible pigmented area of hair, present at birth. Indeed, Strain *et al.* (1992), Strain (2003a) and Juraschko *et al*. (2003a, 2003b) demonstrated that patched Dalmatians were less likely to be deaf than unpatched animals and that the absence of iris pigmentation had a significant association with hearing disorder in this breed. It is suspected that the observed association of CSD with pigmentation is related to the extreme white piebald allele s^w, seen for example in Dalmatians, Bullterriers, English Setters, Dogo Argentinos, or to the merle allele M, which is found in breeds such as the Collie,

Shepherd Dog, Dachshund or Great Dane (Strain 1996). The question of the causal relationship between CSD and the s^w allele still remains controversial. This allele is suspected of producing white colouration by acting on the differentiation and/or migration of melanocyte precursor cells from the neural crest during embryogenesis. It is likely that a strong expression of s^w results in a reduction of melanocytes, e.g. in the eye and inner ear, thus leading to blue eyes and deafness, and that a weak expression of s^w results in a pigmented area, such as the patches seen in Dalmatians (Strain, 2003a). In fact, mouse models have shown melanocytes to be essential for normal hearing function (see below). The assumption that CSD is related to white producing genes and not to other coat pigmentation varieties was supported by the results of Juraschko *et al.* (2003a, 2003b) and Strain (2003a).
Although several investigations showed a significant association between sex and deafness in Dalmatians, with females having a higher incidence (Famula *et al*., 2001; Greibrokk, 1994, Holliday *et al*., 1992; Wood and Lakhani, 1997, 1998), Anderson *et al*. (1968) found a higher incidence in males, and other analyses indicated no significant gender difference in the prevalence of deafness (Juraschko *et al*., 2003b; Strain *et al*., 1992). It is difficult to explain why gender effects were seen in the former studies but not in the latter. Juraschko *et al*. (2003b) assumed that the different prevalences observed were due to different sample routines between studies. This may also be applicable to gender association. It has been suggested (Famula *et al*., 2001) that these differences may reflect the fact that BAER testing is voluntary in the USA, or that founder effects are being seen in the UK. Relative geographical restriction effects, litter effects and other variables may also have had an impact (Strain, 2003a).

Inheritance patterns

For most of the affected dog breeds, neither the proof of inheritance nor the mode of inheritance of CSD has been provided. Deafness in the Doberman Pinscher and in nervous Pointer dogs is known to be transmitted by a simple recessive mechanism (Steinberg *et al*., 1994; Wilkes and Palmer, 1992). The disorder has been reported to have an autosomal recessive mechanism in the Rottweiler, Bull Terrier and Pointer, as well. But the latter suggestion has to be questioned because those reports were published before the possibility of BAER testing and the ability to detect unilaterally deaf dogs (Strain, 2003b). Unsurprisingly, most available data concerning the mechanism of inheritance are for Dalmatian dogs. Even

though several studies have demonstrated that CSD in Dalmatians is an inherited disease, the conclusions differ as to the inheritance mechanism and the number of contributing genes. Neither a dominant nor a recessive simple Mendelian mode of transmission could be proven by pedigree analyses. Anderson *et al*. (1968) proposed an X-linked gene with different expressivity and Nelson (1991) assumed that two different genes were responsible which require at least one dominant allele at each locus. Other hypotheses on inheritance included a model of two interacting recessive loci with incomplete penetrance (Strain *et al*., 1992), an autosomal multifactorial recessive gene with incomplete penetrance (Greibrokk, 1994), and polygenic determination (Famula *et al*., 1996). In a subsequent study Famula *et al*. (2000) postulated that a major single recessive gene may play an important role in the transmission of deafness, but they could not completely explain the inheritance of the disease. A segregation analysis of the German Dalmatian dog population showed that a mixed monogenic-polygenic model with a major recessive gene and eye colour as covariate best explained the segregation of affected animals in the pedigrees (Juraschko *et al*., 2003a, 2003b). Of course, as described for phenotypical and gender associations, population differences, different breeding standards or sampling routines may be possible explanations for the deviating conclusions. Moreover, it must be recalled that statistical results are always dependent on the model used, on the parameters that have been included and on the sample size.

Histopathological findings as candidate gene approach

Studying the pathology of a genetic disease often allows the selection of good candidate genes. Candidate genes may be suggested on the basis of a close functional relationship to a gene known to be involved in a similar disease. Such genes could encode different interacting components in the same metabolic or developmental pathway. A good candidate gene should have an expression pattern consistent with the disease phenotype. The expression does not need to be restricted to the affected tissue, as there are many examples of widely expressed genes causing tissue-specific diseases, but the candidate should at least be expressed at the time and in the place where the pathology occurs (Strachan and Read, 2001a). For these reasons the following section contains a description of the histopathological findings of canine deafness.

Congenital sensorineural hearing impairment can be recognised in dogs at four to eight weeks of age (Strain, 1996), while histological studies of deaf Dalmatians have shown that the degeneration of the inner ear structures begins as early as one day after birth and is histologically clearly evident by four weeks of age (Johnsson *et al*., 1973). The histological pattern of CSD in many affected dog breeds is known as cochleo-saccular degeneration, commonly known as Scheibe dysplasia with preservation of the pars superior of the membranous labyrinth and an unremarkable bony labyrinth. Most histological studies have been performed with Dalmatian dogs (Anderson *et al*., 1968; Branis and Burda, 1985; Ferrara and Halnan, 1983; Igarashi *et al*., 1972; Johnsson *et al*., 1973; Niparko and Finger, 1997; Mair, 1976; Rouse *et al*., 1984). However, reports of such examinations are rare for other breeds, or refer to findings from only a few animals or even of a single affected individual (Coppens *et al*., 2000, 2001, 2003; Hiraide and Paparella, 1988; Igarashi *et al*., 1972). Cochleo-saccular degeneration has been noted as a dynamic process closely related to the animals' age. The histological findings in deaf dogs generally include degeneration of the stria vascularis, which is a vascular bed located at the outer margin of the cochlear duct of the inner ear, sagging of the Reissner's membrane followed by the collapse of the cochlear duct, degeneration of hair cells and supporting cells of the organ of Corti, and collapse of the saccule. Secondary loss of spiral ganglion cells of the cochlear nerve is seen in later stages. In contrast, Coppens et al. (2001) found a severe ganglion cell degeneration in a bilaterally deaf Rottweiler puppy only 4.5 months old.

Mair (1976) found two different types of pathological changes in the tectorial membrane simultaneously occurring in Dalmatian dogs: the tectorial membrane was frequently found to be rolled up and retracted into the inner spiral sulcus and also appeared outlined as a prominent, shelf-like structure. Others reported an at least partial calcification of the tectorial membrane in one or both affected ears in different dog breeds (Hiraide and Paparella, 1988; Rouse *et al*., 1984).

Rouse *et al.* (1984) found a mixture of mineral deposits (calcite otoconia and apatite sherulites) on the saccular otoconial membrane in Dalmatians. In individual cases there were crystals resembling otoconia on the surface of the stria vascularis or transparent aggregate crystals on the utricular otoconial membrane. The authors could, however, only speculate about the origin and significance of the crystals in those individual cases. Although several investigators have proposed that the degenerative changes in the Dalmatian organ of Corti are

secondary to the atrophy of the stria vascularis, Mair (1976) suggested that the genetically determined hair cell degeneration could occur in the presence of a histologically normal stria. Recent studies have shown that deafness in Dobermans results from a direct loss of cochlear hair cells without any effects on the stria vascularis (Strain, 2003b). The cause of the strial degeneration is as yet unknown. But histological studies in mouse mutants (Steel *et al*., 1987) with cochleo-saccular abnormalities and in deaf Dalmatians (Lurie, 1948) demonstrated the absence of strial melanocyte-like cells, which appear to be vital for normal stria vascularis development and function (Steel and Barkway, 1989). These findings could be a possible explanation for the association between deafness and pigmentation defects.

Human deafness in comparison to canine hearing loss

Over the past decade it has become increasingly clear how far structural and functional homologies at the gene level extend across even very distantly related species. Extensive homologies can be detected between human genes and genes in zebrafish, Drosophila and even in yeast. Humans and dogs are more closely related than the species mentioned above, and various genes that are known to be involved in human deafness have already been identified. The comparison of human and canine deafness therefore provides the possibility of a candidate gene approach to clarify CSD in dogs.

Deafness is the most common human sensory disorder world-wide (Bitner-Glindzicz, 2002), with approximately one in one thousand children born with a serious permanent hearing impairment, and about 60% of people over 70 suffering from progressive hearing loss. Approximately one-half of the cases of childhood deafness is thought to be caused by a single gene defect. However, the nature of the genetic contribution to progressive hearing loss has not yet been clearly defined (Morton, 1991; Steel and Kros, 2001).

Clinically, hearing impairment may be associated with other disorders (syndromic), or it may be the only symptom (non-syndromic). In dogs, reports of syndromic deafness are generally rare, and there has always been disagreement as to whether syndromic canine deafness occurs at all. An exception is the Doberman Pincher, which has shown to suffer from sensorineural deafness accompanied by transient vestibular dysfunction (Wilkes and Palmer, 1992). Rouse *et al.* (1984) reported a Dalmatian dog with abnormal locomotor behaviour. Another syndromic form of hearing loss in Dalmatian dogs has been described by Schaible (1986),

who found a significantly higher incidence of CSD in dogs that form purine uroliths. Unlike canine deafness, in humans several hundred forms of syndromes with hearing loss have been documented (Gorlin *et al*., 1995). One is the human Waardenburg syndrome (WS), which manifests itself with sensorineural deafness and pigmentation defects in the iris, hair and skin. The WS is classified into four types, depending on the presence or absence of additional symptoms, which are caused by mutations in the five genes *EDN3*, *EDNRB*, *MITF*, *PAX3* and *SOX10*, respectively. These genes are known to be expressed in the neural crest (*EDN3*, *EDNRB, PAX3*, *SOX10*) or directly in the melanocytes (*MITF*), and are, inter alia, involved in migration, differentiation or survival of melanocytes, respectively (Bondurand *et al*., 2000). Therefore, Brenig *et al*. (2003) and Rak *et al*. (2003) suspected that these genes are suitable candidates for pigment-associated deafness in many affected dog breeds.

Non-syndromic forms of deafness account for 70% of patients with prelingual hearing loss. Among these non-syndromic forms it is estimated that the predominant form is autosomal recessive inheritance, with up to 75 to 80% of cases, followed by dominant (20 to 25%) and X-linked inheritance (1 to 1.5%). Mitochondrial mutations with maternally transmitted hearing loss may also occur (Cohen and Gorlin, 1995; Morton, 1991; Petit, 1996).

In 70% of the cases with human hereditary deafness the histological pattern is known as cochleo-saccular degeneration (Lalwani *et al*., 1997), which is similar to that of canine deafness.

Several studies attempted to estimate the number of loci for human deafness in various populations, with results ranging from less than ten to several thousand (Chung *et al*., 1959; Costeff and Dar, 1980; Brownstein *et al*. 1991). Over the past ten years, significant progress has been made in the identification of deafness gene localisations, and at present 70 different chromosomal loci for non-syndromic hearing loss have been mapped; but not even half of the genes responsible for these loci have as yet been cloned (Table 3). This extreme heterogeneity of human deafness often hampered genetic studies because many different genetic forms of hearing loss give rise to similar clinical phenotypes, and, conversely, mutations in the same gene can result in a variety of clinical phenotypes. Evidence for the first statement has for example been given by a study of Adato et al. (2000), who investigated the molecular basis of hearing impairment in four Druze families practising endogamous marriage. Thus it was expected that a single mutation would account for the hearing impairment in all these families. But the results showed that at least four different genes were involved. An example

for the latter statement are different deafness phenotypes caused by different mutations in the gene *GJB2* which encodes a gap junction protein in the inner ear. *GJB2* is known to cause dominant and recessive non-syndromic forms of prelingual sensorineural deafness. But other mutations in the same gene are also responsible for autosomal dominant keratoderma combined with the sensorineural deafness known as Vohwinkel syndrome (Petersen, 2002). Syndromic hearing impairment tends to be genetically less heterogeneous than non-synromic, but more than one locus has been identified for several syndromes (Table 3) (Van Camp and Smith, 2003). As mentioned above, in some cases the same gene may be responsible for both dominant and recessive non-syndromic deafness (e.g. *GJB2*, *GJB6*, *TECTA*), and it has also been shown that both syndromic and non-syndromic phenotypes can result from different mutations in the same gene (e.g. *GJB2*, *CDH23*, *COL11A2*, *MYO7A*, *SLC26A4*).

Table 3. Number of loci and cloned genes for non-syndromic and syndromic deafness in humans (Hereditary Hearing Loss Homepage 2003).

Mode of inheritance [a]/ Syndromic deafness[b]	**Symbol**	**Number of loci**	**Number of cloned genes**
Autosomal dominant	DFNA	31	12
Autosomal recessive	DFNB	28	13
Autosomal dominant and recessive	DFNA/DFNB	6	6
X-linked	DFN	5	1
Syndromic deafness	-	28	24

[a] Mode of inheritance is given for non-syndromic deafness.

[b] Syndromes inluded: Alport syndrome, Brachio-Oto-Renal syndrome, Norrie Disease, Pendred, Stickler, Teacher Collins, Usher and Waardenburg syndromes.

Many of these genes are expressed in the inner ear and are involved in cochlear physiology and development. Unsurprisingly, a wide variety of molecules has now been implicated in the causation of deafness in humans and mice, including transcription factors, motor molecules

(e.g. unconventional myosins), extracellular matrix components, gap and tight junctions, ion channels and ion channel activators, and many more (Table 4). And as the different histological findings within one canine breed and in different dog breeds have shown, one could assume the same situation in dogs, as well.

Table 4. Genes involved human non-syndromic deafness[a].

Predicted function/Class of protein	**Genes**
Unconventional myosin	*MYO3A*, *MYO6*, *MYO7A*, *MYO15A*, *MYH9*
Cytogenesis and cell polarity	*DIAPH1*
Extracellular matrix component	*COCH*, *COL11A2*, *TECTA*, *STRC*
Gap junction/tight junction	*GJA1*, *GJB2*, *GJB3*, *GJB6*, *CLDN14*
Ion channel/ion channel activator	*SLC26A4*, *KCNQ4, TMPRSS3*
Transcription factor/transcriptional activator	*POU4F3*, *POU3F4*, *EYA4*
Membrane protein	*TMC1*, *WFS1*, *CDH23*
Vesicle membrane fusion	*OTOF*
Unknown	*DFNA5*

[a] This table does not provide a complete listing.

Comparative genomics

Although mapping and identifying genes have been highly successful approaches in single, large families with dominant inheritance, in large consanguineous families, and in isolated populations, genetic testing and diagnosis remains difficult in small human families and in sporadic cases of deafness. And although the BAER test is a reliable method for identifying unilaterally and bilaterally deaf dogs, it does not seem to be an effective way of clearly reducing the incidence of deafness in affected breeds, particularly in a recessive mode of inheritance, so that hearing dogs can still be genetic carriers. There is no doubt that the future challenge in the study of human and canine deafness will be to identify and analyse the

function of (additional) deafness-causing genes using high-density genome screens and the analysis of candidate genes.

Comparative genomics has made many valuable contributions to disease gene identification (Meisler, 1996; Brown and Steel 1994). But in respect to deafness, there is a large number of mouse mutants with no obvious human homologue, human deafness genes have been localised or identified with no available equivalent mouse model (Steel and Kros, 2001) and, moreover, there are often discrepancies between the phenotypes of mice and humans carrying the same gene defect (Shastry, 2000).

In the past, it has repeatedly been shown that the physiology, disease presentation and clinical response of dogs often closely mimic human diseases (Ostrander and Kruglyak, 2000), and, as indicated above, hearing impairment seems to be no exception. An advantage of a dog model for human deafness would be that there probably is a large resource of new, spontaneous mutations due to the many dog breeds affected by congenital deafness and due to the numerous, very similar histological findings that have been described so far. A reliable diagnosis of deafness in dogs can be achieved using the BAER test without having to euthanise the animals before making a histological diagnosis, as is the common practice with mouse models. An additional advantage of a dog model would be that histological examinations are easier to perform than with rodent models. Moreover, the dog, with its short generation interval, relatively high number of progeny, and relatively long lifespan (in comparison to rodent models), offers the possibility of generating back-cross matings to optimise a pedigree for linkage analysis, as for example in the discovery of the cause of canine narcolepsy (Lin *et al*., 1999).

In recent years, there have been major advances in the canine genome map. The current version of the comprehensive radiation hybrid map of the canine genome is composed of 3270 markers including 1596 microsatellites, 900 cloned gene sequences and ESTs, 668 bacterial artificial chromosome ends and 106 STSs. The localisation of dog gene markers on this map revealed 85 conserved segments between dogs and humans (Guyon *et al*., 2003). Nevertheless, at present the canine genome map is still relatively poor in localised genes. With the microsatellite-based markers, however, it is now possible to carry out whole genome scans for deafness in dogs. Although linkage can in theory be detected between loci that are up to 40 cM apart, the amount of data required to do this is prohibitive. Ten meioses are sufficient to give evidence of linkage if there are no recombinants, but 85 meioses would be

needed to give equally strong evidence of linkage if the recombination fraction was 0.3 (Strachan and Read, 2001b). Obtaining enough family material to test this amount of required meioses may be difficult. Thus mapping requires markers no larger than about 20 cM across the genome. Given the canine genome lengths that have been calculated so far (2700±100 cM) (Neff *et al.*, 1999), this means that we need a minimum of about 140 highly informative markers. Allowing whole genome screens for imperfect informative markers, at least 280 markers are necessary. In the second step of the linkage study, much denser maps are needed, with average spacing of markers down to 1 cM, to progress from the initial mapping of the disease to a special canine chromosome until the identification of the exact chromosomal region is achieved. Once a canine deafness locus has been successfully mapped and compared with the conserved chromosomal region(s) between dogs and humans, a promising candidate gene would be sought that shows appropriate expression and/or appropriate function. Another strategy for identifying deafness-causing genes in one species (dogs or humans), which avoids the necessity of a complete genome scan, would be to select candidate genes that have already been shown to be deafness-causing in the other species. The respective cDNA or a PCR product of a coding region of this candidate gene can be used for screening the heterologous genomic DNA library in order to isolate genomic DNA clones containing the candidate gene. Once the candidate gene has been mapped, one could look for closely localised markers in the maps available. It is additionally possible to screen the isolated genomic DNA clones in order to identify new and directly gene-associated markers. These markers could be used for linkage studies in families segregating for deafness. Using these methods, it would be possible to detect and to analyse yet unknown deafness-causing genes in both humans and dogs. Of the breeds of dogs that suffer from congenital hearing impairment, the Dalmatian, with its high prevalence of deafness, seems to be the most suitable breed for performing such linkage studies. If linkage to a deafness-causing gene has been detected in Dalmatians it would then have to be discovered if this result could be applied to the other affected dog breeds.

The genes causing human Waardenburg syndrome are only examples of a few suitable candidate genes for deafness in dogs, and many more genes with mutations known to result in human cochleo-saccular degeneration could possibly be involved in canine CSD. In fact, within the last years the first steps in the mapping (Breen *et al*., 2001; Drögemüller *et al*., 2002; Krempler *et al*. 2000; Kuiper *et al*., 2002; Rak *et al*., 2002a, 2002b, 2003; Schmutz *et*

al. 2001) and analysis (Brenig *et al*., 2003) of candidate genes for canine deafness have already been performed based on current knowledge of human deafness. A total of 24 potential candidate genes have now been mapped to 16 different canine chromosomes. The identification and mapping of these genes provides the resource of molecular genetic studies for CSD in dogs. It may be assumed that additional candidate genes for canine CSD will be identified and analysed in the next few years.

Conclusions

Remarkable progress has been made in the past few years in identifying deafness genes in man and mouse. There have likewise been major advances in the canine genome map, and great strides made towards understanding the histological and genetic background of canine hearing loss. Comparative genomics can now be used as a powerful and very effective approach towards unravelling the genetic basis of canine and human deafness. If causal mutations for canine CSD are identified, breeding strategies can be developed to reduce the incidence in affected dog breeds while gaining new insights into the molecular mechanism of auditory function, and possibly translating these basic findings into therapeutic strategies.

Acknowledgements

Simone G. Rak is supported by a grant from the Gesellschaft zur Förderung Kynologischer Forschung (GKF) e.V., Bonn, Germany.

References

ADATO, A., RASKIN, L., PETIT, C. & BONNE-TAMIR, B. (2000): Deafness heterogeneity in a Druze isolate from the Middle East: novel OTOF and PDS mutations, low prevalence of GJB2 35delG mutation and indication for a new DFNB locus. *European Journal of Human Genetics* 8, 437-42.

ANDERSON, H., HENRICSON, B., LUNDQUIST, P. G., WEDENBERG, E. & WERSALL, J. (1968): Genetic hearing impairment in the Dalmatian dog. An audiometric, genetic and morphologic study in 53 dogs. *Acta Oto-Laryngologica Supplement* 232, 1-34.

BITNER-GLINDZICZ, M. (2002): Hereditary deafness and phenotyping in humans. *British Medical Bulletin* 63, 73-94.

BONDURAND, N., PINGAULT, V., GOERICH, D. E., LEMORT, N., SOCK, E., CAIGNEC, C. L., WEGNER, M. & GOOSSENS, M. (2000): Interaction among SOX10, PAX3 and MITF, three genes altered in Waardenburg syndrome. *Human Molecular Genetics* 9, 1907-17.

BRANIS, M. & BURDA, H. (1985): Inner ear structure in the deaf and normally hearing Dalmatian dog. *Journal of Comparative Pathology* 95, 295-99.

BREEN, M., JOUQUAND, S., RENIER, C., MELLERSH, C. S., HITTE, C., HOLMES, N. G., CHERON, A., SUTER, N., VIGNAUX, F., BRISTOW, A. E., PRIAT, C., MCCANN, E., ANDRÉ, C., BOUNDY, S., GITSHAM, P., THOMAS, R., BRIDGE, WL., SPRIGGS, H. F., RYDER, E. J., CURSON, A., SAMPSON, J., OSTRANDER, E. A., BINNS, M. M. & GALIBERT, F. (2001): Chromosome-Specific Single-Locus FISH Probes Allow Anchorage of an 1800-Marker Integrated Radiation-Hybrid/Linkage Map of the Domestic Dog Genome to All Chromosomes. *Genome Research* 11, 1784-95.

BRENIG, B., PFEIFFER, I., JAGGY, A., KATHMANN, I., BALZARI, M., GAILLARD, C. & DOLF, G. (2003): Analysis of the 5' region of the canine PAX3 gene and exclusion as a candidate for Dalmatian deafness. *Animal Genetics* 34, 47-50.

BROWN, S. D. & STEEL, K. P. (1994): Genetic deafness-progress with mouse models. *Human Molecular Genetics* 3, 1453-6.

BROWNSTEIN, Z., FRIEDLANDER, Y., PERITZ, E. & COHEN, T. (1991): Estimated number of loci for autosomal recessive severe nerve deafness within the Israeli Jewish population, with implications for genetic counseling. *American Journal of Medical Genetics* 41, 306-12.

CHUNG, C. S., ROBISON, O. W. & MORTON, N. E. (1959): A note of deaf mutism. *Annals of Human Genetics* 23, 357-66.

COHEN, M. M. & GORLIN, R. J. (1995). Epidemiology, etiology, and genetic patterns, In *Hereditary hearing loss and is syndromes*, eds. Gorlin, R. J., Toriello, H. V., Cohen, M. M., pp. 9-21, Oxford: Oxford University Press.

COPPENS, A. G., RESIBOIS, A. & PONCELET, L. (2000): Bilateral deafness in a Maltese terrier and a great pyrenean puppy: inner ear morphology. *Journal of Comparative Pathology* 122, 223-8.

COPPENS, A. G., KISS, R., HEIZMANN, C. W., DELTENRE, P. & PONCELET, L. (2001): An original inner ear neuroepithelial degeneration in a deaf Rottweiler puppy. *Hearing Research* 161, 65-71.

COPPENS, A. G., STEINBERG, S. A. & PONCELET, L. (2003): Inner ear morphology in a bilaterally deaf Dogo Argentino pup. *Journal of Comparative Pathology* 128, 67-70.

COSTEFF, H. & DAR, H. (1980): Consanguinity analysis of congenital deafness in Northern Israel. *American Journal of Human Genetics* 32, 64-8.

DRÖGEMÜLLER, C., RAK, S. G., KUIPER, H., LEEB, T., QUIGNON, P., GALIBERT, F. & DISTL, O. (2002): Assignment of the canine tectorin alpha gene (TECTA) to CFA5q12→q13 by FISH and confirmation by radiation hybrid mapping. *Cytogenetic and Genome Research* 97, 140A.

EGER, C. E. & LINDSAY, P. (1997): Effects of otitis on hearing in dogs characterised by brainstem auditory evoked response testing. *The Journal of Small Animal Practice* 38, 380-6.

FAMULA, T. R., OBERBAUER, A. M. & SOUSA C. A. (1996): A threshold model analysis of deafness in Dalmatians. *Mammalian Genome* 7, 650-3.

FAMULA, T. R., OBERBAUER, A. M. & SOUSA, C. A. (2000): Complex segregation analysis of deafness in Dalmatians. *American Journal of Veterinary Research* 61, 550-53.

FAMULA, T. R., OBERBAUER, A. M. & WILLIAMS, D. C. (2001): Gender effects in hearing loss in Dalmatians. *Preventive Veterinary Medicine* 48, 15-24.

FERRARA, M. L. & HALNAN, C. R. (1983): Congenital structural brain defects in the deaf Dalmatian. *The Veterinary Record* 112, 344-6.

GORLIN, R. J. (1995): Genetic hearing loss with no association abnormalities. In *Hereditary hearing loss and its syndromes*, eds. Gorlin, R. J., Toriello, H. V., Cohen, M. M., pp. 43-61, Oxford: Oxford University Press.

GREIBROKK, T. (1994): Hereditary Deafness in the Dalmatian: Relationship to Eye and Coat Color. *Journal of the American Animal Hospital Association* 30, 170-76.

GUYON, R., LORENTZEN, T. D., HITTE, C., KIM, L., CADIEU, E., PARKER, H. G., QUIGNON, P., LOWE, J. K., RENIER, C., GELFENBEYN, B., VIGNAUX, F., DEFRANCE, H. B., GLOUX, S., MAHAIRAS, G. G., ANDRÉ, C., GALIBERT, F. & OSTRANDER, E. A. (2003): A 1-Mb resolution radiation hybrid map of the canine genome. *Proceedings of the National Academy of Sciences of the United States of America* 100, 5296-301.

HIRAIDE, F. & PAPARELLA M. M. (1988): Histopathology of the temporal bones of deaf dogs. *Auris Nasus Larynx* 15, 97-104.

HOLLIDAY, T. A., NELSON, H. J., WILLIAMS, D. C. & WILLITS, N. (1992): Unilateral and bilateral brainstem auditory-evoked response abnormalities in 900 Dalmatian dogs. *Journal of Veterinary Internal Medicine* 6, 166-74.

IGARASHI, M., ALFORD, B. R., SAITO, R., COHN, A. M. & WATANABE, T. (1972): Inner ear anomalies in dogs. *The Annals of Otology, Rhinology, and Laryngology* 81, 249-55.

JOHNSSON, L. G., HAWKINS, J. E., MURASKI, A. A. & PRESTON, R. E. (1973): Vascular anatomy and pathology of the cochlea in Dalmatian dogs. In *Vascular Disorders and Hearing Defects,* ed. deLorenzo, A. J. D., pp. 249-95, Baltimore: University Park Press.

JURASCHKO, K., MEYER-LINDENBERG, A., NOLTE, I. & DISTL, O. (2003a): A regressive model analysis of congenital sensorineural deafness in German Dalmatian dogs. *Mammalian Genome* 14, 547-54.

JURASCHKO, K., MEYER-LINDENBERG, A., NOLTE, I. & DISTL, O. (2003b): Analysis of systematic effects on congenital deafness in German Dalmatian Dogs. *The Veterinary Journal* 166, 164-9.

KREMPLER, A., BREEN, M. & BRENIG, B. (2000): Assignment of the canine paired-box 3 (PAX3) gene to chromosome 37q16→q17 by in situ hybridization. *Cytogenetics and Cell Genetics* 90, 66-7.

KUIPER, H., RAK, S. G., DRÖGEMÜLLER, C., LEEB, T., QUIGNON, P., GALIBERT, F. & DISTL, O. (2002): Assignment of the canine cadherin related 23 gene (CDH23) to chromosome 4q12→q13 by fluorescence in situ hybridization and radiation hybrid mapping. *Cytogenetic and Genome Research* 97, 140B.

LALWANI, A. K., LINTHICUM, F. H., WILCOX, E. R., MOORE, J. K., WALTERS, F. C., SAN AGUSTIN, T. B., MISLINSKI, J., MILLER, M. R., SINNINGER, Y., ATTAIE, A. & LUXFORD, W. M. (1997): A five-generation family with late-onset progressive hereditary hearing impairment due to cochleo-saccular degeneration. *Audiology & Neuro-otology* 2, 139-54.

LIN, L., FARACO, J., LI, R., KADOTANI, H., ROGERS, W., LIN, X., QIU, X., DE JONG, P. J., NISHINO, S. & MIGNOT, E. (1999): The sleep disorder canine narcolepsy is caused by a mutation in the hypocretin (orexin) receptor 2 gene. *Cell* 98, 365-76.

LURIE, M. D. (1948): The membranous labyrinth in the congenital deaf Collie and Dalmatian dog. *Laryngoscope* 58, 279-87.

MAIR, I. W. (1976): Hereditary deafness in the Dalmatian dog. *Archives of Oto-Rhino-Laryngology* 212, 1-14.

MEISLER, M. H. (1996): The role of the laboratory mouse in the human genome project. *American Journal of Human Genetics* 59, 764-71.

MORTON, N. E. (1991): Genetic epidemiology of hearing impairment. *Annals of the New York Academy of Sciences* 630, 16-31.

MUHLE, A. C., JAGGY, A., STRICKER, C., STEFFEN, F., DOLF, G., BUSATO, A., KORNBERG, M., MARISCOLI, M., SRENK, P. & GAILLARD, C. (2002): Further contributions to the genetic aspect of congenital sensorineural deafness in Dalmatians. *The Veterinary Journal* 163, 311-8.

NEFF, M. W., BROMAN, K. W., MELLERSH, C. S., RAY, K., ACLAND, G. M., AGUIRRE, G. D., ZIEGLE, J. S., OSTRANDER, E. A. & RINE, J. (1999). A second-generation genetic linkage map of the domestic dog, Canis familiaris. *Genetics* 151, 803-20.

NELSON, H. J. (1991): Deafness in Dalmatians – 1986. Spots of news. *Journal of the British Dalmatian Club* 362, 10-12.

NIPARKO, J. K. & FINGER, P. A. (1997): Cochlear nucleus cell size changes in the Dalmatian: model of congenital deafness. *Otolaryngology Head and Neck Surgery* 117, 229-35.

OSTRANDER, E. A. & KRUGLYAK, L. (2000): Unleashing the canine genome. Genome Research 10, 1271-74.

PATTERSON, D. F. (2000): Companion animal medicine in the age of medical genetics. *Journal of Veterinary Internal Medicine* 14, 1-9.

PETERSEN, M. B. (2002): Non-syndromic autosomal-dominant deafness. *Clinical Genetics* 62, 1-13.

PETIT, C. (1996): Genes responsible for human hereditary deafness: symphony of a thousand. *Nature Genetics* 14, 385-91.

RAK, S. G., DRÖGEMÜLLER, C., KUIPER, H., LEEB, T., QUIGNON, P., ANDRÉ, C. & DISTL, O. (2002a): Comparative mapping of the canine diaphanous homologue 1 (Drosophila) gene (DIAPH1) to CFA2q23-q24.2. *Animal Genetics* 33, 389-90.

RAK, S. G., DRÖGEMÜLLER, C., KUIPER, H., LEEB, T., QUIGNON, P., ANDRÉ, C. & DISTL O. (2002b): Cloning and chromosomal localization of MYO15A to chromosome 5 of the dog (Canis familiaris). *Chromosome Research* 10, 407-10.

RAK, S. G., DRÖGEMÜLLER, C., LEEB, T., QUIGNON, P., ANDRÉ, C., SCOTT, A., BREEN, M. & DISTL O. (2003): Chromosomal assignment of 20 candidate genes for canine congenital sensorineural deafness by FISH and RH mapping. *Cytogenetic and Genome Research* 101, 130-35.

RAWITZ, B. (1896): Gehörorgan und Gehirn eines weißen Hundes mit blauen Augen. *Morphologische Arbeiten* 6, 545-53.

ROUSE, R. C., JOHNSSON, L. G., WRIGHT, C. G. & HAWKINS JR., J. E. (1984): Abnormal otoconia and calcification in the labyrinths of deaf Dalmatian dogs. *Acta Oto-Laryngologica* 98, 61-71.

SCHAIBLE, R. H. (1986): Genetic predisposition to purine uroliths in Dalmatian dogs. *The Veterinary Clinics of North America. Small Animal Practice* 16, 127-31.

SCHMUTZ, S. M., MOKER, J. S., YUZBASIYAN-GURKAN, V., ZEMKE, D., SAMPSON, J., LINGAAS, F., DUNNER, S. & DOLF, G. (2001): DCT and EDNRB map to DogMap linkage group L07. *Animal Genetics* 32, 321.

SHASTRY, B. S. (2000): Mammalian cochlear genes and hereditary deafness. *Microbial & Comparative Genomics* 5, 61-9.

SIMS, M. H. & MOORE R. E. (1984): Auditory-evoked response in the clinically normal dog: early latency components. *American Journal of Veterinary Research* 45, 2019-27.

STEEL, K. P., BARKWAY, C. & BOCK, G. R. (1987): Strial dysfunction in mice with cochleo-saccular abnormalities. *Hearing Research* 27, 11-26.

STEEL, K. P. & BARKWAY, C. (1989): Another role for melanocytes: their importance for normal stria vascularis development in the mammalian inner ear. *Development* 107, 453-63.

STEEL, K. P. & KROS, C. J. (2001): A genetic approach to understanding auditory function. *Nature Genetics* 27, 143-9.

STEFFEN, F. & JAGGY, A. (1998): Deafness and its diagnosis in dogs and cats. *Schweizer Archiv für Tierheilkunde* 140, 397-04.

STEINBERG, S. A., KLEIN, E., KILLENS, R. L. & UHDE, T. W. (1994): Inherited deafness among nervous Pointer dogs. *The Journal of Heredity* 85, 56-59.

STRACHAN, T. & READ, A. P. (2001a): Identifying human disease genes. In *Human Molecular Genetics*, ed. Kingston, F., pp. 351-75, Bath, UK: The Bath Press Ltd.

STRACHAN, T. & READ, A. P. (2001b): Genetic mapping of mendelian characters. In *Human Molecular Genetics*, ed. Kingston, F., pp. 269-82, Bath, UK: The Bath Press Ltd.

STRAIN, G. M. (1996): Aetiology, prevalence and diagnosis of deafness in dogs and cats. *The British Veterinary Journal* 152, 17-36.

STRAIN, G. M. (1999): Congenital Deafness and Its Recognition. *The Veterinary clinics of North America. Small animal practice* 29, 895-07.

STRAIN, G. M. (2003a): Deafness prevalence and pigmentation and gender associations in dog breeds at risk. *The Veterinary Journal* 167, in press (http://www.lsu.edu/deafness/VetJDeaf2003.pdf.)

STRAIN, G. M. (2003b): Genetics of Deafness in Dogs. Deafness in Dogs and Cats. Homepage http://www.lsu.edu/deafness/genetics.htm.

STRAIN, G. M., KEARNEY, M. T., GIGNAC, I. J., LEVESQUE, D.C., NELSON, H. J., TEDFORD, B. L. & REMSEN, L. G. (1992): Brainstem auditory-evoked potential assessment of congenital deafness in Dalmatians: associations with phenotypic markers. *Journal of Veterinary Internal Medicine* 6, 175-82.

STRAIN, G. M., GREEN, K. D., TWEDT, A. C. & TEDFORD, B. L. (1993): Brain stem auditory evoked potentials from bone stimulation in dogs. *American Journal of Veterinary Research* 54, 1817-21.

VAN CAMP, G. & SMITH, R. J. H. (July, 2003): Hereditary Hearing Loss Homepage. http://dnalab-www.uia.ac.be/dnalab/hhh/.

WILKES, M. K. & PALMER, A. C. (1992): Congenital deafness and vestibular deficit in the Doberman. *The Journal of Small Animal Practice* 33, 218.

WOOD, J. L. N. & LAKHANI, K. H. (1997): Prevalence and Prevention of Deafness in the Dalmatian - Assessing the Effect of Parental Hearing Status and Gender using Ordinary Logistic and Generalized Random Litter Effect Models. *The Verterinary Journal* 154, 121-33.

WOOD, J. L. N. & LAKHANI, K. H. (1998): Deafness in Dalmatians: does sex matter? *Preventive Veterinary Medicine* 36, 39-50.

Chapter 3

Chromosomal assignment of 20 candidate genes for canine congenital sensorineural deafness by FISH and RH mapping

Simone G. Rak, Cord Drögemüller, Tosso Leeb, Pascale Quignon, Catherine André, Allyson Scott, Matthew Breen and Ottmar Distl

Published in: Cytogenetic and Genome Research 101 (2003) 130-135

Chromosomal assignment of 20 candidate genes for canine congenital sensorineural deafness by FISH and RH mapping

Abstract

The analysis of inherited diseases in the domestic dog (*Canis familiaris*) provides a resource for the continued use of this species as a model system for human diseases. Many different dog breeds are affected by congenital sensorineural deafness. Since mutations in various genes have already been found causative for sensorineural hearing impairment in humans or mice, 20 of these genes were considered as candidates for deafness in dogs. For each of the candidate genes a canine BAC clone was isolated by screening with heterologous human or murine cDNA probes. The gene-containing BAC clones were physically assigned to the canine genome by FISH and the BAC derived STS-markers were positioned with the RHDF5000 panel on the canine RH map. The mapping data, which confirm the established conservation of synteny between canine and human chromosomes, provide a resource for further association studies in segregating canine populations and the basis for new insights into this common canine and human disease.

Introduction

Many genetic disorders in humans and domestic dogs (*Canis familiaris*) demonstrate a high level of clinical and molecular similarity. As a consequence the domestic dog is used widely as an animal model in the study of human diseases. The limiting step in revealing parallels at the gene level of other hereditary canine and human diseases has been the cloning and charactrization of canine disease genes (Ostrander et al., 2000).

Congenital sensorineural deafness has been reported for approximately 54 different breeds of dogs (Strain, 1996). The incidence of this inherited congenital anomaly is highest in Dalmatian dogs of which 16.5-30% exhibit unilateral or bilateral hearing loss (Famula et al., 1996; Juraschko et al., 2003; Muhle et al., 2002; Wood and Lakhani, 1997). In humans, hereditary deafness is highly heterogeneous with over 100 loci identified so far (Read, 2000). In approximately 70% of cases with human hereditary deafness (Lalwani et al., 1997) and in many affected dog breeds the histological pattern is known as cochleo-saccular degeneration, commonly known as Scheibe dysplasia (Strain, 1996).

Due to the complex phenotype and the suspected heterogeneity of canine deafness, 20 genes were selected as candidates for deafness in dogs. These 20 candidates include the five genes that are mutated in the human Waardenburg syndrome (WS) that manifests with sensorineural deafness and pigmentation defects in iris, hair and skin (endothelin 3 [EDN3], endothelin receptor type B [EDNRB], microphthalmia-associated transcription factor [MITF], paired box gene 3 [PAX3], SRY [sex determining region Y]-box 10 [SOX10]) (Bondurand et al., 2000), and an additional 15 genes that are involved in human non-syndromic sensorineural deafness. The functions of these 15 deafness-causing genes are diverse and include gap junctions and tight junctions (connexin 43 [GJA1], connexin 26 [GJB2], connexin 30 [GJB6], claudin 14 [CLDN14]), ion channels (solute carrier family 26, member 4 [SLC26A4]) and ion channel activators (transmembrane protease, serine 3 [TMPRSS3]). Included are also unconventional myosins (myosin VI [MYO6], myosin VIIA [MYO7A], myosin, heavy polypeptide 9, non-muscle [MYH9]), transcription factors (POU domain, class 4, transcription factor 3 [POU4F3], eyes absent homolog 4 [Drosophila] [EYA4]) as well as extracellular matrix components (coagulation factor C homolog, cochlin [COCH], collagen, type XI, alpha 2 [COL11A2]) and genes with unknown or only suspected functions (deafness, autosomal dominant 5 [DFNA5], otoferlin [OTOF]). In human and mouse it has been shown that mutations in members of the connexin gene family are associated with non-syndromic deafness. Three of these genes, GJB2, GJB6 and GJA1, are encoding gap junction proteins in the inner ear, which are participating in the recycling of potassium to the cochlear endolymph (Liu et al., 2001). In mice it has been demonstrated that *Cldn14*, encoding for a protein of tight junctions, is expressed in the sensory epithelium of the organ of Corti. Tight junctions in the cochlear duct are thought to compartmentalize the endolymph and provide structural support for the auditory neuroepithelium (Wilcox et al., 2001). Mutations in the human ion channel gene SLC26A4 cause both syndromic and non-syndromic deafness. The expression pattern involves several regions thought to be important for endolymphatic fluid resorption in the inner ear (Everett et al., 1999). The TMPRSS3 gene, mutated in human deafness, is expressed in the spiral ganglion, the cells supporting the organ of Corti and the stria vascularis and activates an epithelial sodium channel (Guipponi et al., 2002). In addition, three members of the myosin gene family are also implicated in deafness: mutations in MYO6 and MYO7A cause a fusion and degeneration of stereocilia respectively and subsequent progressive loss of hair cells (Friedman et al., 1999). The MYH9 gene is involved in human non-syndromic

sensorineural deafness caused by cochleo-saccular degeneration (Lalwani et al., 2000). The expression of both *Pou4f3* and *Otof* has been mainly detected in mouse inner ear hair cells. While POU4F3 is known to encode for a transcription factor, the sequence homologies and predicted structure of otoferlin, the protein encoded by OTOF, suggest the involvement of this gene in vesicle membrane fusion (Vahava et al., 1998; Yasunaga et al., 1999). Although the EYA proteins play a role in the regulation of early embryonic development, the EYA4 gene also seems to be important post developmentally for the continued function of the mature organ of Corti (Wayne et al., 2001). The cochlin gene (COCH) is expressed almost exclusively in the inner ear. Affected individuals with mutations in COCH were found to have mucopolysaccharide depositions in the channels of the cochlear and vestibular nerves causing strangulation and degeneration of dentritic fibers (Robertson et al., 1998). Electron microscopy of the tectorial membrane of mice with targeted disruption of *Col11a2* revealed loss of organization of the collagen fibrils and a unique ultrastructural malformation of the inner ear architecture (McGuirt et al., 1999). Mutations in DFNA5 that is expressed in human fetal cochlea cause autosomal dominant progressive hearing loss in humans but little is known about the physiologic function yet (Van Laer et al., 1998).

The aim of this study was the chromosomal assignment of 20 potential candidate genes for sensorineural deafness in dogs by fluorescence *in situ* hybridization and by radiation hybrid mapping.

Material and methods

Screening the BAC library and partial BAC sequence analysis

Human or murine IMAGE cDNA clones for the selected genes were obtained from the German Human Genome Resource Center-/Primary Database (http://www.rzpd.de/) (Table 1). High density BAC filters from the canine RPCI-81 BAC library (Li et al., 1999) were hybridized according to the RPCI protocols (http://www.chori.org/bacpac/) with ^{32}P–labeled inserts of the IMAGE clones. DNA of positive BAC clones was isolated using the Qiagen plasmid maxi kit (Qiagen, Hilden, Germany), *Pst* I fragments were separated by gel-electrophoresis on 0.8% agarose gels and southern blotting was used to transfer the fragments to a nylon membrane. Confirmation of the identity of isolated clones was made initially by hybridization with the inserts of IMAGE cDNA clones, using the ECL^{TM} direct labeling and

detection kit (Amersham Biosciences, Freiburg, Germany). The BAC termini were sequenced with the ThermoSequenase kit (Amersham Biosciences, Freiburg, Germany) on a LI-COR 4200L-2 automated sequencer. The BAC DNA was then restricted with different enzymes and separated on 0.8% agarose gels. The resulting fragments were cloned into the polylinker of pGEM®-4Z (Promega, Mannheim, Germany) and sequenced as mentioned above. All generated sequences were then compared with database sequences using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/) and the Ensembl Genome Server (http://www.ensembl.org/) as a means to verify sequence identity between the canine and human/mouse sequences. To determine the insert sizes, the BAC DNA was digested with *Not* I and then fractionated on 1% agarose pulsed field gels.

Fluorescence in situ hybridization (FISH)

Two hundred nanograms of each of the 20 canine BAC DNAs were labeled for FISH using nick translation to incorporate either Spectrum Red dUTP, Spectrum Green dUTP, Spectrum Orange dUTP or DEAC-dUTP as described previously (Breen et al., 1999a). Twenty five nanograms of each labeled clone were co-precipitated in the presence of differentially labeled clones and 10 µg of sonicated canine genomic DNA as competitor. The probe plus competitor precipitate was resuspended in 15 µl of hybridization buffer comprising 50% deionized formamide, 2 x SSC, 10% dextran sulfate and 0.1% Tween-20. Probes were hybridized to canine metaphase spreads and post-hybridization washing was performed as described elsewhere (Breen et al., 1999b). Chromosomes were counterstained in 80 ng/ml 4',6'-diaminidino-2-phenylindole (DAPI) and mounted in antifade solution (Vectashield, Vector Laboratories, Peterborough, U.K.).

Image acquisition and chromosome assignment

Images were acquired and processed using a multi-color FISH workstation comprising a fluorescence microscope (Axioplan 2ie, Zeiss) equipped with narrow pass filter sets for the visualization of Spectrum Red, Spectrum Green, Spectrum Orange, DEAC and DAPI and a cooled CCD camera (CoolSnapHQ, Photometrics, Tuscon, AZ, USA) both driven by dedicated software (SmartCapture 2.3.1 Digital Scientific, Cambridge, U.K.). The digital image of each DAPI stained metaphase spread was processed using a high-pass spatial filter

to reveal enhanced DAPI bands. Each clone was assigned to a chromosome band according to the DAPI banded nomenclature of Breen et al. (1999a).

Radiation hybrid (RH) mapping

For each candidate gene, one positive BAC clone was selected and the BAC end sequences were used as STS-markers after designing a pair of PCR primers for RH mapping (Table 2). Amplification of the markers was tested using standard conditions on dog and hamster DNA and on a mixture (1:3) of dog and hamster DNA. PCR reactions were carried out in PTC-200 MJ Research PCR machines with the following program: 8 min 94 °C, followed by 20 cycles of 30 sec 94 °C, 30 sec maximum annealing temperature (AT) (see Table 2 for AT) decreasing by 0.5 °C per cycle, 30 sec 72 °C and 15 cycles of 30 sec 94 °C, 30 sec maximum AT minus 10 °C, 30 sec 72 °C and a final extension of 2 min at 72 °C.

All tested STS-markers were dog specific and could be readily typed on the radiation hybrid panel RHDF5000 composed of 118 cell lines (Vignaux et al., 1999) using the above PCR conditions. PCR products were loaded on 2% agarose gels. Products were visualized under UV light, images were recorded and results were scored in terms of present, absent or ambiguous in the 118 hybrid cell lines.

Statistical analysis

The typing data, obtained in duplicate, were computed onto the radiation hybrid map (Breen et al., 2001), using the Multimap package (Matise et al., 1994). On this map, the markers were placed by a two-point analysis, i.e. close to the markers of the map presenting the best LOD score with each marker.

Results and discussion

For each of the 20 selected candidate genes (Table 1) at least one canine BAC clone was isolated by screening the RPCI-81 BAC library. The identity of the clones was confirmed by gene-specific heterologous hybridization of the isolated canine BAC DNA and subsequent DNA sequencing of BAC fragments.

Table 1. Details of the selected human candidate genes.

Symbol	Gene name	Locus[a, b]/WS type[a]	Human location	Human or murine cDNA clone ID[c]
CLDN14	claudin 14	DFNB29	21q22.3	IMAGp998D036999
COCH	coagulation factor C homolog, cochlin	DFNA9	14q11.2-q13	IRALp962H015
COL11A2	collagen, type XI, alpha 2	DFNA13	6p21.3	DKFZp434E1172
DFNA5	deafness, autosomal dominant 5	DFNA5	7p15	IMAGp998N0710189
EDN3	endothelin 3	WS type IV	20q13.2-q13.3	IMAGp998B10127
EDNRB	endothelin receptor type B	WS type IV	13q22	IMAGp998B135498
EYA4	eyes absent homolog 4 (Drosophila)	DFNA10	6q23	IMAGp998K203083
GJA1	gap junction protein, alpha 1, 43kD (connexin 43)	[d]	6q21-q23.2	IRAKp961P0416
GJB2	gap junction protein, beta 2, 26k (connexin 26)	DFNA3/DFNB1	13q11-q12	IMAGp998G171219
GJB6	gap junction protein, beta 6 (connexin 30)	DFNA3/DFNB1	13q12	IMAGp998N228514
MITF	microphthalmia-associated transcription factor	WS type II	3p14.1-p12.3	IMAGp998O2410022
MYH9	myosin, heavy polypeptide 9, non-muscle	DFNA17	22q12.3-q13.1	IMAGp998K046429
MYO6	myosin VI	DFNA22/DFNB37	6q13	IRALp962O1619
MYO7A	myosin VIIA	DFNA11/DFNB2	11q13.5	IMAGp998B085668
OTOF	otoferlin	DFNB9	2p23.1	IMAGp998E21159
PAX3	paired box gene 3 (Waardenburg syndrome 1)	WS type I/WS type III	2q35-q37	IMAGp998H095214
POU4F3	POU domain, class 4, transcription factor 3	DFNA15	5q31	IMAGp998M169565
SLC26A4	solute carrier family 26, member 4	DFNB4	7q31	IMAGp998C175333
SOX10	SRY (sex determining region Y)-box 10	WS type IV	22q13	IMAGp998C149751
TMPRSS3	transmembrane protease, serine 3	DFNB8/DFNB10	21q22.3	IMAGp998F175323

[a] Adapted from Van Camp G and Smith (2003).

[b] Autosomal recessive loci are designated DFNB, autosomal dominant loci DFNA.

[c] http://www.rzpd.de/

[d] To our knowledge no deafness locus has been determined for this autosomal recessive gene.

Table 2. Assignment of candidate genes for deafness in dogs. For each gene the accession number of a BAC fragment, BAC insert size, PCR primers and optimized conditions are shown. The canine genome locations determined by FISH and RH mapping are presented.

Gene	EMBL Acc.	BAC insert size (kb)	Primer sequences (5'→3`)	PCR product (bp)	Max[a] AT (°C)	Canine location		Linked to marker[b]	LOD score
						FISH	RH		
CLDN14	AJ557548	160	F: CCTGATTAGGGGCATCAGAA R: TCAGATTTCAGGCCAGGTTT	207	61	31q15	31	HSD20B	11.7
COCH	AJ557549	100	F: CACTTCACTGTTTTGCCCTGT R: TTGAGAGCGACAAGGAGGAT	169	61	8q12-q13	8	E04007	23.6
COL11A2	AJ557550	180	F: TTGCATCACCATCATTAGGC R: TGATGTCTAGAACCTCCAATATTAAAC	296	61	12q11-q12	12	DLADQA	27.3
DFNA5	AJ557551	150	F: GCCGATAGGTGGCCATATTA R: GGCAAAGTTCCCATCTCTCA	340	61	14q21.1	14	REN289L09	13.2
EDN3	AJ557552	190	F: GAGGAACGAAAACACGGGTA R: CATGGTTCTGAGGGTCATCC	199	61	24q24-q25	24	REN272I16	13.7
EDNRB	AJ557553	225				22q21-q22	22[c]		
EYA4	AJ557554	165	F: CCCCGGGTTCCTATCACTAA R: GAAAATGCGGCAATACACAA	222	61	1q13.3	1	REN135K06	20.1
GJA1	AJ557555	185	F: TCCTCCTTCCATGTCTCTGC R: CTGGTGGAATGAGGTTCTCAG	194	61	1q24-q25	1	C02509	18.1
GJB2	AJ557556	175	F: TGGCACAGAGTGATGGAGAC R: ACTGCCCCTTTTCACTGCTA	210	61	25q12-q21	25	FH2324	10.2
GJB6	AJ557557	200	F: GGAAGGAAGCTTGGGAAATC R: ATCAAAATGGCTGGAACAGG	196	61	25q12-q21	25	FH2324	11.2
MITF	AJ557558	150	F: TATGGGTCCTGAGAGCTGGT R: GGAGCAGAGACTGGAAGCTG	200	65	20q13	20	REN100J13	15.8
MYH9	AJ459259	145	F: GGAGCTTCCTCTTCGCTTCT R: TGCAGAAACCCCATTTCTCT	210	61	10q23.2	10	FH2293	co-localized
MYO6	AJ557559	150	F: TGGCCCAAACTATGAATAACG R: ATCAACGAATGCCATTTTGG	213	59	12q21	12	REN148F07	26.3
MYO7A	AJ557560	240	F: GGCTTCCTTTCCTTCTCAGG R: CCCCAACATCTATGGGTCTG	204	61	21q13.3-q21	21	AHTH298	24.2
OTOF	AJ557561	200	F: GGTTGGCATCAGGTTATGGT R: TTCTGATGCTGGTCTGAGGA	198	65	17q13	17	C02604	16.9

Table 2. Continued

Gene	EMBL Acc.	BAC insert size (kb)	Primer sequences (5'→3`)	PCR product (bp)	Max[a] AT (°C)	Canine location		Linked to marker[b]	LOD score
						FISH	RH		
PAX3	AJ557565	190	F: GCACACAACCCTTCCAGAAT R: CCGTGAACAACACAAGATGG	222	61	37q16-q17[d]	37	AHT135	21.4
POU4F3	AJ557562	140	F: GCTCTCCAAGGGTGTAGCAG R: AGCCCCATGTTTCTCAAATC	201	61	2q21	2	C02.466	co-localized
SLC26A4	AJ557563	110	F: AGCCTCAAATTGGAAAAGCA R: ATTAGGTGCTTTGGGACACG	184	61	18q21	18	REN249N22	22
SOX10	AJ557566	145	F: GAGGCAGCTTCCAGAAGAGA R: ATCCCCTTCACGAGTCACAT	202	65	10q21-q23	10	REN108O17	19
TMPRSS3	AJ557564	145	F: GCTTGTGGGTTCGCTGTATT R: GCCCTGCAGTCTCTGAACTC	194	61	31q15	31	AHTH246	24.8

[a] AT = annealing temperature.

[b] These markers are included in the canine RH map available at http://www-recomgen.univ-rennes1.fr/doggy.html.

[c] Breen et al. (2001).

[d] See also Krempler et al. (2000).

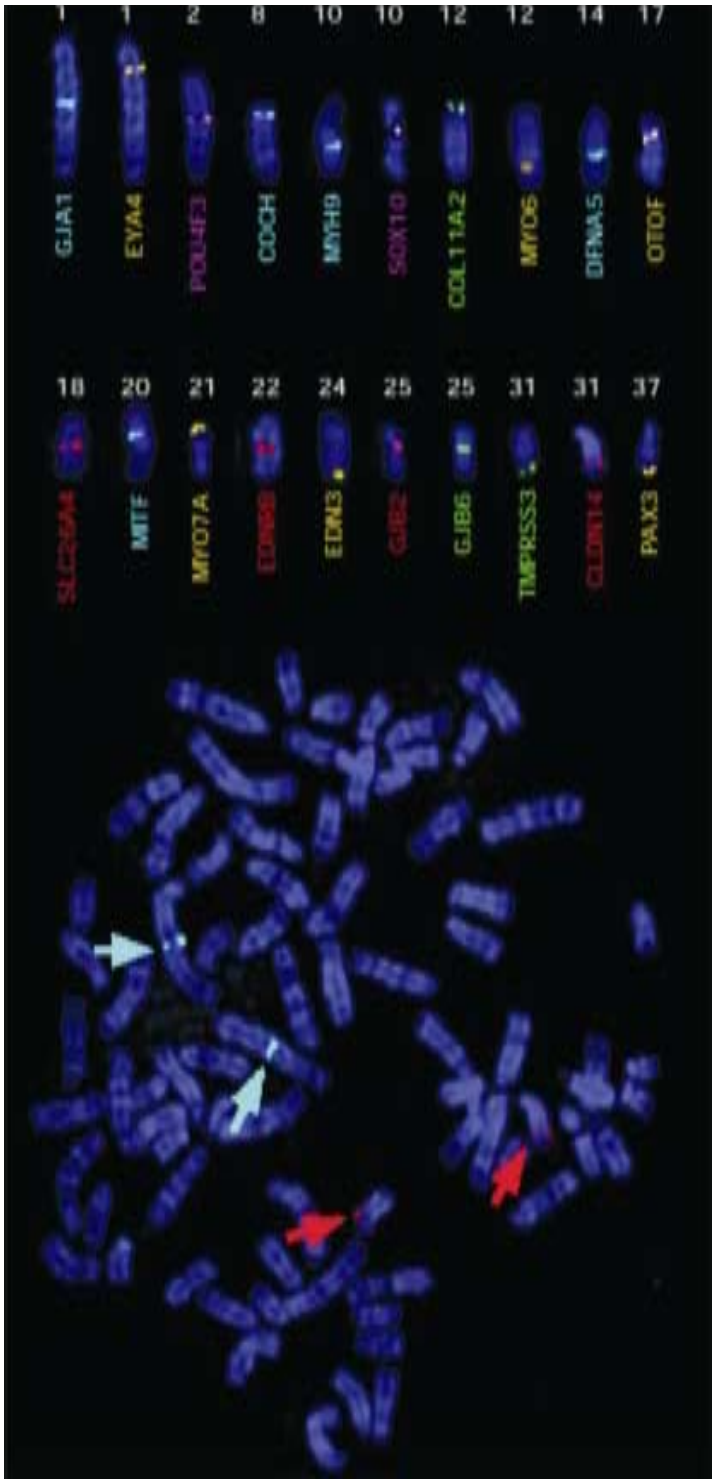


Figure 1. Fluorescence *in situ* hybridization assignment of 20 canine candidate genes for canine deafness. The whole canine metaphase spread shows the co-hybridization of a DEAC labeled BAC clone containing GJA1 (blue signal) and a spectrum red labeled BAC clone containing CLDN14 (red signal) to canine chromosomes 1 and 31 respectively. The panel of single chromosomes at the top of the figure shows the signals derived from the hybridization of the 20 different BAC clones with the corresponding gene name and the chromosome number indicated below and above the chromosome respectively.

BLAST searches of these canine sequences revealed significant matches ($E < 10^{-5}$) to the corresponding human genes or to the human chromosomal regions flanking the corresponding genes (EMBL Acc. nos. are shown in Table 2).

The chromosomal localizations of the gene-containing BAC clones, determined by radiation hybrid mapping and by fluorescence *in situ* hybridization, are shown in Table 2 and in Figure 1. For all 20 genes, concordant results have been obtained with these two independent methods.

The genes GJB2 and GJB6 were both mapped by FISH to CFA25q12-q21 and were closely linked by RH mapping to the same marker, FH2324. BAC fingerprinting indicated that the isolated canine BAC clones for these two genes overlap. The close genomic organization between GJB2 and GJB6 is also found in human (Table 1), where it has been demonstrated that the complex DFNB1 (Deafness B1) locus situated on HSA 13q12 contains both GJB2 and GJB6 (del Castillo et al., 2002) and encompasses a region of about 35 kb (http://www.ensembl.org/). The two genes CLDN14 and TMPRSS3 have been mapped in the same interval on CFA31q15. This close location is in agreement with the location of the two orthologous human genes that are both situated on HSA21q22.3. The myosin genes MYO6, MYO7A and MYH9 are localized on three different canine chromosomes, CFA12, 21 and 10. The remaining seven candidate genes that are involved in human non-syndromic deafness are dispersed on seven different canine chromosomes and are all tightly linked to existing markers in the integrated canine genome map (LOD scores of at least 13.2 (Table 2)).

Of the five genes that cause the human WS, the EDNRB gene has previously been genetically mapped to CFA22 by Schmutz et al. (2001). Moreover Breen et al. (2001) mapped EDNRB by RH mapping to CFA22, an assignment we have now confirmed by FISH (Table 2). We have located the PAX3 gene on CFA 37q16-q17 by fluorescence *in situ* hybridization in concordance with the FISH results of a different BAC clone for this gene (Krempler et al., 2000). Our BAC derived STS marker for PAX3 has also been positioned at the telomeric end on CFA37 by RH mapping and is closely linked to the microsatellite AHT135. Although Brenig et al. (2003) have excluded PAX3 to be a responsible gene for deafness in Dalmatians (predominantly in Swiss Dalmatian populations), this gene could remain as a candidate in other Dalmatian populations. Indeed, different founder effects may occur in several geographically isolated lines leading to the existence of different mutations in one gene or in different genes. The three other WS genes EDN3, MITF and SOX10 have been located on

CFA24, 20 and 10 respectively and each was linked to a mapped microsatellite with LOD scores of at least 13.7 (Table 2).

The genes that are mutated in the human WS were selected as candidates because the WS phenotype, where the deafness is associated with pigmentation defects, seems to be similar to the phenotype of most affected dog breeds (Strain and Tedford, 1996). Both Juraschko et al. (2003) and Strain et al. (1992) have demonstrated that patched Dalmatians are less likely to be deaf than unpatched animals and blue-eyed Dalmatians are more likely to be affected from hearing impairment than brown-eyed animals.

The mechanism of inheritance for canine deafness is unknown for most dog breeds (Strain, 1999) and for those breeds for which the mode of inheritance has been analyzed it is not quite clear how many genes are involved. For example the segregation of the disease phenotype in a German Dalmatian dog population was best explained by a recessive major gene model with polygenic components including eye color as a covariate (Juraschko et al., 2003). Famula et al. (1996) showed a large additive genetic variation, and were later able to detect a single recessive gene transmission in addition to polygenic inheritance in congenital sensorineural deafness of American Dalmatian dogs (Famula et al., 2000). Another hypothesis on inheritance in Dalmatian dogs included two recessive genes of different origin (Strain et al., 1992). Deafness in the Doberman Pinscher, which is accompanied by transient vestibular dysfunction, and in nervous Pointer dogs is only known to be transmitted by a simple recessive mechanism (Wilkes and Palmer, 1992; Steinberg et al., 1994).

The assignments observed in this study, are all in good agreement with known synteny data between human and canine chromosomes on the dog genome map (Breen et al., 2001). In terms of comparative mapping the data presented herein support the high precision of the available human-dog comparative map.

With the isolation and mapping of the canine BAC clones containing the selected candidate genes, we provide the basis for future association studies. To clarify the role of these genes in congenital sensorineural deafness in dogs, segregating families can now be genotyped with known closely linked microsatellite markers.

Acknowledgements

This project and Simone G. Rak are supported by a grant from the Gesellschaft zur Förderung Kynologischer Forschung (GKF) e.V., Bonn, Germany. Pascale Quignon is supported by funds from the Conseil Regional de Bretagne, France. Matthew Breen is supported by funds from the American Kennel Club Canine Health Foundation, USA.

References

BONDURAND N, PINGAULT V, GOERICH DE, LEMORT N, SOCK E, CAIGNEC CL, WEGNER M & GOOSSENS M (2000): Interaction among SOX10, PAX3 and MITF, three genes altered in Waardenburg syndrome. Hum Mol Genet 9, 1907-1917.

BREEN M, BULLERDIEK J & LANGFORD CF (1999a): The DAPI banded karyotype of the domestic dog (*Canis familiaris*) generated using chromosome-specific paint probes. Chromosome Res 7, 401-406.

BREEN M, THOMAS R, BINNS MM, CARTER NP & LANGFORD CF (1999b): Reciprocal chromosome painting reveals detailed regions of conserved synteny between the karyotypes of the domestic dog (*Canis familiaris*) and human. Genomics 61, 145-155.

BREEN M, JOUQUAND S, RENIER C, MELLERSH CS, HITTE C, HOLMES NG, CHERON A, SUTER N, VIGNAUX F, BRISTOW AE, PRIAT C, MCCANN E, ANDRÉ C, BOUNDY S, GITSHAM P, THOMAS R, BRIDGE WL, SPRIGGS HF, RYDER EJ, CURSON A, SAMPSON J, OSTRANDER EA, BINNS MM & GALIBERT F (2001): Chromosome-Specific Single-Locus FISH Probes Allow Anchorage of an 1800-Marker Integrated Radiation-Hybrid/Linkage Map of the Domestic Dog Genome to All Chromosomes. Genome Res 11, 1784-1795.

BRENIG B, PFEIFFER I, JAGGY A, KATHMANN I, BALZARI M, GAILLARD C & DOLF G (2003): Analysis of the 5' region of the canine PAX3 gene and exclusion as a candidate for Dalmatian deafness. Anim Genet 34, 47-50.

DEL CASTILLO I, VILLAMAR M, MORENO-PELAYO MA, DEL CASTILLO FJ, ALVAREZ A, TELLERIA D, MENENDEZ I & MORENO F (2002): A deletion involving the connexin 30 gene in nonsyndromic hearing impairment. N Engl J Med 346, 243-249.

EVERETT LA, MORSLI H, WU DK & GREEN ED (1999): Expression pattern of the mouse ortholog of the Pendred's syndrome gene (Pds) suggests a key role for pendrin in the inner ear. Proc Natl Acad Sci 96, 9727-9732.

FAMULA TR, OBERBAUER AM & SOUSA CA (1996): A threshold model analysis of deafness in Dalmatians. Mamm Genome 7, 650-653.

FAMULA TR, OBERBAUER AM & SOUSA CA (2000): Complex segregation analysis of deafness in Dalmatians. Am J Vet Res 61, 550-553.

FRIEDMAN TB, SELLERS JR & AVRAHAM KB (1999): Unconventional myosins and the genetics of hearing loss. Am J Med Genet 89, 147-157.

GUIPPONI M, VUAGNIAUX G, WATTENHOFER M, SHIBUYA K, VAZQUEZ M, DOUGHERTY L, SCAMUFFA N, GUIDA E, OKUI M, ROSSIER C, HANCOCK M, BUCHET K, REYMOND A, HUMMLER E, MARZELLA PL, KUDOH J, SHIMIZU N, SCOTT HS, ANTONARAKIS SE & ROSSIER BC (2002): The transmembrane serine protease (TMPRSS3) mutated in deafness DFNB8/10 activates the epithelial sodium channel (ENaC) in vitro. Hum Mol Genet 11, 2829-2836.

JURASCHKO K, MEYER-LINDENBERG A, NOLTE I & DISTL O (2003): A regressive model analysis of congenital sensorineural deafness in German Dalmatian dogs. Mamm Genome 14, 547-554.

KREMPLER A, BREEN M & BRENIG B (2000): Assignment of the canine paired-box 3 (PAX3) gene to chromosome 37q16→q17 by in situ hybridization. Cytogenet Cell Genet 90, 66-67.

LALWANI AK, LINTHICUM FH, WILCOX ER, MOORE JK, WALTERS FC, SAN AGUSTIN TB, MISLINSKI J, MILLER MR, SINNINGER Y, ATTAIE A & LUXFORD WM (1997): A five-generation family with late-onset progressive hereditary hearing impairment due to cochleo-saccular degeneration. Audiol Neurootol 2, 139-154.

LALWANI AK, GOLDSTEIN JA, KELLEY MJ, LUXFORD W, CASTELEIN CM & MHATRE AN (2000): Human nonsyndromic hereditary deafness DFNA17 is due to a mutation in nonmuscle myosin MYH9. Am J Hum Genet 67, 1121-1128.

LI R, MIGNOT E, FARACO J, KADOTANI H, CANTANESE J, ZHAO B, LIN X, HINTON L, OSTRANDER EA, PATTERSON DF & DE JONG PJ (1999): Construction and characterization of an eightfold redundant dog genomic bacterial artificial chromosome library. Genomics 58, 9-19.

LIU XZ, XIA XJ, ADAMS J, CHEN ZY, WELCH KO, TEKIN M, OUYANG XM, KRISTIANSEN A, PANDYA A, BALKANY T, ARNOS KS & NANCE WE (2001): Mutations in GJA1 (connexin 43) are associated with non-syndromic autosomal recessive deafness. Hum Mol Genet 10, 2945-2951.

MATISE TC, PERLIN M & CHAKRAVARTI A (1994): Automated construction of genetic linkage maps using an expert system (MultiMap): A human genome linkage map. Nat Genet 6, 384-390.

MCGUIRT WT, PRASAD SD, GRIFFITH AJ, KUNST HP, GREEN GE, SHPARGEL KB, RUNGE C, HUYBRECHTS C, MUELLER RF, LYNCH E, KING MC, BRUNNER HG, CREMERS CW, TAKANOSU M, LI SW, ARITA M, MAYNE R, PROCKOP DJ, VAN CAMP G & SMITH RJ (1999): Mutations in COL11A2 cause non-syndromic hearing loss (DFNA13). Nat Genet 23, 413-419.

MUHLE AC, JAGGY A, STRICKER C, STEFFEN F, DOLF G, BUSATO A, KORNBERG M, MARISCOLI M, SRENK P & GAILLARD C (2002): Further contributions to the genetic aspect of congenital sensorineural deafness in Dalmatians. Vet J 163, 311-318.

OSTRANDER EA, GALIBERT F & PATTERSON DF (2000): Canine genetics comes of age. Trends Genet, 16, 117-124.

READ AP (2000): Hereditary deafness: lessons for developmental studies and genetic diagnosis. Eur J Pediatr 159, 232-235.

ROBERTSON NG, LU L, HELLER S, MERCHANT SN, EAVEY RD, MCKENNA M, NADOL JB JR, MIYAMOTO RT, LINTHICUM FH JR, LUBIANCA NETO JF, HUDSPETH AJ, SEIDMAN CE, MORTON CC & SEIDMAN JG (1998): Mutations in a novel cochlear gene cause DFNA9, a human nonsyndromic deafness with vestibular dysfunction. Nat Genet 20, 299-303.

SCHMUTZ SM, MOKER JS, YUZBASIYAN-GURKAN V, ZEMKE D, SAMPSON J, LINGAAS F, DUNNER S & DOLF G (2001): DCT and EDNRB map to DogMap linkage group L07. Anim Genet 32, 321.

STEINBERG SA, KLEIN E, KILLENS RL & UHDE TW (1994): Inherited deafness among nervous pointer dogs. J Hered 85, 56-59.

STRAIN GM (1996): Aetiology, prevalence and diagnosis of deafness in dogs and cats. Br Vet J 152, 17-36.

STRAIN GM (1999): Congenital Deafness and Its Recognition. Small Animal Practice - Special Issue: Pediatrics. http://www.lsu.edu/deafness/VetClinNA.htm.

STRAIN GM, KEARNEY MT, GIGNAC IJ, LEVESQUE DC, NELSON HJ, TEDFORD BL & REMSEN LG (1992): Brainstem auditory-evoked potential assessment of congenital deafness in Dalmatians: associations with phenotypic markers. J Vet Intern Med 6, 175-182.

STRAIN GM & TEDFORD BL (1996): Deafness prevalence and associated phenotypic markers in dog breeds with high risk. J Vet Intern Med 10, 190.

VAHAVA O, MORELL R, LYNCH ED, WEISS S, KAGAN ME, AHITUV N, MORROW JE, LEE MK, SKVORAK AB, MORTON CC, BLUMENFELD A, FRYDMAN M, FRIEDMAN TB, KING MC & AVRAHAM KB (1998): Mutation in transcription factor POU4F3 associated with inherited progressive hearing loss in humans. Science 279, 1950-1954.

VAN CAMP G & SMITH RJH (April, 2003): Hereditary Hearing Loss Homepage. http://dnalab-www.uia.ac.be/dnalab/hhh/.

VAN LAER L, HUIZING EH, VERSTREKEN M, VAN ZUIJLEN D, WAUTERS JG, BOSSUYT PJ, VAN DE HEYNING P, MCGUIRT WT, SMITH RJ, WILLEMS PJ, LEGAN PK, RICHARDSON GP & VAN CAMP G (1998): Nonsyndromic hearing impairment is associated with a mutation in DFNA5. Nat Genet 20, 194-197.

VIGNAUX F, HITTE C, PRIAT C, CHUAT JC, ANDRÉ C & GALIBERT F (1999): Construction and optimization of a dog whole-genome radiation hybrid panel. Mamm Genome 10, 888-894.

WAYNE S, ROBERTSON NG, DECLAU F, CHEN N, VERHOEVEN K, PRASAD S, TRANEBJARG L, MORTON CC, RYAN AF, VAN CAMP G & SMITH RJ (2001): Mutations in the transcriptional activator EYA4 cause late-onset deafness at the DFNA10 locus. Hum Mol Genet 10, 195-200.

WILCOX ER, BURTON QL, NAZ S, RIAZUDDIN S, SMITH TN, PLOPLIS B, BELYANTSEVA I, BEN-YOSEF T, LIBURD NA, MORELL RJ, KACHAR B, WU DK, GRIFFITH AJ, RIAZUDDIN S & FRIEDMAN TB (2001): Mutations in the gene encoding tight junction claudin-14 cause autosomal recessive deafness DFNB29. Cell 104, 165-172.

WILKES MK & PALMER AC (1992): Congenital deafness and vestibular deficit in the doberman. J Small Anim Pract 33, 218.

WOOD JLN & LAKHANI KH (1997): Prevalence and Prevention of Deafness in the Dalmatian - Assessing the Effect of Parental Hearing Status and Gender using Ordinary Logistic and Generalized Random Litter Effect Models. J Vet 154, 121-133.

YASUNAGA S, GRATI M, COHEN-SALMON M, EL-AMRAOUI A, MUSTAPHA M, SALEM N, EL-ZIR E, LOISELET J & PETIT C (1999): A mutation in OTOF, encoding otoferlin, a FER-1-like protein, causes DFNB9, a nonsyndromic form of deafness. Nat Genet 21, 363-369.

Chapter 4

Comparative mapping of the canine diaphanous homolog 1 (Drosophila) gene (DIAPH1) to CFA2q23-q24.2

Simone G. Rak, Cord Drögemüller, Heidi Kuiper, Tosso Leeb, Pascale Quignon, Catherine André and Ottmar Distl

Published in: Animal Genetics 33 (2002) 389-90

Comparative mapping of the canine diaphanous homolog 1 (Drosophila) gene (*DIAPH1*) to CFA2q23-q24.2

Source/description

Mutation of the human homolog of the Drosophila diaphanous gene (*DIAPH1*) leads to autosomal dominant, fully penetrant, non-syndromic sensorineural progressive hearing loss (DFNA1) in humans (Lynch et al., 1997). DFNA1 has been localized to the human chromosome 5q31 (Leon et al.,1992). The biological role of human diaphanous in hearing is likely to be the regulation of actin polymerization in hair cells of the cochlea in the inner ear (Lynch et al., 1997). Congential non-syndromic sensorineural deafness has been reported in more than 54 different breeds of dogs (Strain, 1996). Histological examinations showed that in some cases a degeneration of the auditory hair cells in the cochlea was responsible for canine hearing loss (Johnsson et al., 1973; Mair, 1976). Therefore, the *DIAPH1* gene seems to be a suitable candidate for this inherited canine disease. In an effort to identify and map the *DIAPH1* gene in the dog, a human cDNA clone of *DIAPH1* (IMAGp998B109967) provided by the Ressource Center/Primary Database of the German Human Genome Project (http://www.rzpd.de/) was used to screen the canine RPCI-81 BAC library (http://www.chori.org/bacpac/). A 177 kb genomic DNA clone (RPCI-81_362I5) was isolated after screening the high density BAC filters according to the RPCI protocols (http://www.chori.org/bacpac/) with the ^{32}P–labeled cDNA probe. DNA of the positive BAC clone was isolated using the Qiagen plasmid maxi kit (Qiagen, Hilden, Germany). Sequencing of the canine SP6 BAC terminus (EMBL Acc. AJ459256) revealed a match of 84% identity over 224 bp (BLAST E-value $5x10^{-10}$) to a human genomic DNA clone on chromosome 5 (GenBank AC005753). This BLAST hit is located approximately 115 kb upstream of the first exon of the human *DIAPH1* gene (http://www.ensembl.org/). Due to this BLAST result and the insert size of the canine BAC clone we assume that the clone RPCI-81_362I5 contains about 60 kb of the 5' end of the orthologous canine *DIAPH1* gene.

Radiation hybrid (RH) mapping

A pair of PCR primers for RH mapping (5'-TTGAGCATGCTGTCCTTCAC-3' and 5'-CAGGCTCTCTGCCTCTTCAG-3') was designed from the SP6 sequence of the isolated

BAC clone and a PCR product of 198 bp was obtained from dog genomic DNA. The marker was mapped on the RHDF5000 panel (Vignaux et al., 1999) according to the described protocols (Priat et al., 1998). After typing the RHDF5000 panel in duplicate, the genotyping data were computed with the MultiMap software (Matise et al.,1994) on the latest radiation hybrid map (Breen et al., 2001).

Fluorescence *in situ* hybridization (FISH)

Canine metaphase spreads for FISH on GTG-banded chromosomes were prepared as described (Breen et al., 1999a). The BAC clone containing the canine *DIAPH1* gene was labeled for FISH by nick translation using a Dig-Nick-Translation-Mix (Boehringer Mannheim, Germany). After hybridization over night, signal detection was performed using a Digoxigenin-FITC Detection Kit (Quantum Appligene, Heidelberg, Germany). Identification of the chromosomes was done according to the established GTG-banded and DAPI-banded karyotype of the domestic dog (Breen et al., 1999b; Reimann et al., 1996).

Chromosomal location

The chromosomal location of the canine *DIAPH1* gene was determined by FISH of the BAC clone to metaphase chromosomes. This BAC clone has been assigned to CFA2q23-q24.2. Moreover, the localization of this BAC clone was detected on the canine RHDF5000 radiation hybrid panel. The two-point analysis revealed that *DIAPH1* is linked to the marker *Ren107M12* situated on CFA 2 with a LOD score of 18.8. This chromosomal assignment of *DIAPH1* to CFA2q23-q24.2 confirms the established conservation of synteny between HSA5q31 and CFA2 on the latest dog genome map (Breen et al., 2001).

Acknowledgements

This project and Simone G. Rak are supported by a grant from the Gesellschaft zur Förderung Kynologischer Forschung (GKF) e.V., Bonn. Heidi Kuiper is supported by a grant from the affirmative action program for women of the School of Veterinary Medicine Hannover. Pascale Quignon is supported by funds from the Conseil Regional de Bretagne.

References

BREEN, M., LANGFORD, C. F, CARTER, N. P., HOLMES, N. G., DICKENS, H. F., THOMAS, R., SUTER, N., RYDER, E. J., POPE, M. & BINNS, M. M. (1999a): FISH mapping and identification of canine chromosomes. J Hered 90, 27-30.

BREEN, M., BULLERDIEK, J. & LANGFORD, C. F. (1999b): The DAPI banded karyotype of the domestic dog (*Canis familiaris*) generated using chromosome-specific paint probes. Chromosome Res 7, 401-406.

BREEN, M., JOUQUAND, S., RENIER, C., MELLERSH, C. S., HITTE, C., HOLMES, N. G., CHERON, A., SUTER, N., VIGNAUX, F., BRISTOW, A. E., PRIAT, C., MCCANN, E., ANDRÉ, C., BOUNDY, S., GITSHAM, P., THOMAS, R., BRIDGE, WL., SPRIGGS, H. F., RYDER, E. J., CURSON, A., SAMPSON, J., OSTRANDER, E. A., BINNS, M. M. & GALIBERT, F. (2001): Chromosome-Specific Single-Locus FISH Probes Allow Anchorage of an 1800-Marker Integrated Radiation-Hybrid/Linkage Map of the Domestic Dog Genome to All Chromosomes. Genome Res 11, 1784-1795.

JOHNSSON, L. G., HAWKINS, J. E., MURASKI, A. A. & PRESTON, R. E. (1973): Vascular anatomy and pathology of the cochlea in Dalmatian dogs. In Vascular disorders and hearing defects, ed. deLorenzo, A. J. D., pp. 249-295, Baltimore: University Park Press.

LEON, P. E., RAVENTOS, H., LYNCH, E., MORROW, J. & KING, M. C. (1992): The gene for an inherited form of deafness maps to chromosome 5q31. Proc Natl Acad Sci U S A 89, 5181-5184.

LYNCH, E. D., LEE, M. K., MORROW, J. E., WELCSH, P. L., LEON, P. E. & KING, M. C. (1997): Nonsyndromic deafness DFNA1 associated with mutation of a human homolog of the Drosophila gene diaphanous. Science 278, 1315-1318.

MAIR, I. W. (1976): Hereditary deafness in the Dalmatian dog. Arch Otorhinolaryngol 212, 1-14.

MATISE, T. C., PERLIN, M. & CHAKRAVARTI, A. (1994): Automated construction of genetic linkage maps using an expert system (MultiMap): A human genome linkage map. Nat Genet 6:384-390.

PRIAT, C., HITTE, C., VIGNAUX, F., RENIER, C., JIANG, Z., JOUQUAND, S., CHERON, A., ANDRÉ, C. & GALIBERT, F. (1998): A whole-genome radiation hybrid map of the dog genome. Genomics 54, 361-378.

REIMANN, N., BARTNITZKE, S., BULLERDIEK, J., SCHMITZ, U., ROGALLA, P., NOLTE, I. & RONNE, M. (1996): An extended nomenclature of the canine karyotype. Cytogenet Cell Genet 73, 140-144.

STRAIN, G. M. (1996): Aetiology, prevalence and diagnosis of deafness in dogs and cats. Br Vet J 152, 17-36.

VIGNAUX. F., HITTE, C., PRIAT, C., CHUAT, J. C., ANDRÉ, C. & GALIBERT, F. (1999): Construction and optimization of a dog whole-genome radiation hybrid panel. Mamm Genome 10, 888-894.

Chapter 5

Cloning and chromosomal localization of MYO15A to chromosome 5 of the dog (Canis familiaris)

Simone G. Rak, Cord Drögemüller, Heidi Kuiper, Tosso Leeb, Pascale Quignon, Catherine André and Ottmar Distl

Published in: Chromosome Research 10 (2002) 407-10

Chromosome Research **10**: 407–410, 2002.

Cloning and chromosomal localization of *MYO15A* to chromosome 5 of the dog (*Canis familiaris*)

Simone G. Rak[1†], Cord Drögemüller[1†*], Heidi Kuiper[1], Tosso Leeb[1], Pascale Quignon[2], Catherine André[2], & Ottmar Distl[1]
[1]*Institute of Animal Breeding and Genetics, School of Veterinary Medicine Hannover, Hannover, Germany; Tel: +49-511-9538866; Fax: +49-511-9538582; E-mail: cord.droegemueller@tiho-hannover.de;*
[2]*UMR 6061 CNRS, Génétique et Développement, Faculté de Médecine, 35043 Rennes Cedex, France.*
[†]*S.G.R. and C.D. contributed equally to this work.*
**Correspondence*

Received 4 February 2002; received in revised form and accepted for publication by Wendy Bickmore 18 February 2002

Key words: Canis, chromosome, chromosome painting, dog, gene mapping, myosin XVA

Abstract

Mutations in the myosin XVA gene (*MYO15A*) cause congenital non-syndromic deafness in humans and mice. Therefore, the *MYO15A* gene represents a candidate gene for hereditary hearing loss in dogs. Using a human cDNA to screen a dog BAC library, we isolated a canine BAC clone. Sequencing of the BAC ends confirmed homology to the human gene. To facilitate future linkage studies, we report the physical mapping of the canine *MYO15A* gene to CFA5q23-q24 by FISH and RH mapping.

Introduction

Chromosome painting studies using human chromosome paints on dog chromosomes have been used to identify evolutionarily related regions of the human and dog genomes (Breen *et al.* 1999a). ZOO-FISH analyses generate large comparative mapping data, typically at the level of the chromosome subband. Recently, major advances have been made in the development of an anchored 1800-marker radiation hybrid and linkage map of the canine genome (Breen *et al.* 1999b, 2001). However, the current map contains an average of only eight gene loci per canine chromosome. The latest integrated cytogenetic, RH and comparative map of dog chromosome 5 (CFA5) comprises 14 gene markers (Thomas *et al*. 2001). The short arm of human chromosome 17 (HSA17p) was reported to be homologous to a part of CFA5 (Thomas *et al.* 2001).

The myosin XVA gene (*MYO15A*), known to be located on HSA17p11.2 (Wang *et al.* 1998), was selected for mapping as a candidate gene for congenital non-syndromic hearing loss in dogs. Mutations in the human *MYO15A* are responsible for congenital deafness DFNB3 in humans (OMIM 600316; Wang *et al.* 1998) as well as deafness and vestibular defects in shaker 2 mice. Auditory hair cells in the cochlea of shaker 2 mice have very short stereocilia (OMIM 602666; Probst *et al.* 1998). In some cases, a degeneration of the auditory hair cells in the cochlea has also been responsible for congenital hearing loss in dogs (Johnsson *et al.* 1973, Mair 1976).

Materials and methods

In an effort to identify and map the myosin XVA gene in the dog, a human cDNA clone of *MYO15A* (IMAGp998F184974), provided by the Resource Center/Primary Database of the German Human Genome Project (http://www.rzpd.de/) was used to screen the canine RPCI-81 BAC library. A 180-kb clone (RPCI81_362O13) was isolated after screening the high-density BAC filters according to the RPCI protocols (http://www.chori.org/bacpac/) with the ^{32}P-labeled cDNA probe. DNA of the positive BAC clone was isolated using the Qiagen plasmid maxi kit (Qiagen). Sequencing of BAC termini revealed a significant match between the T7 BAC end sequence (EMBL AJ428857) and the human *MYO15A* gene (GenBank AF051976) of 89% identity over 274 bp of the *MYO15A* 5′UTR. The positive BAC clone was labeled by nick translation using a Nick-Translation-Mix (Boehringer Mannheim). Canine metaphase spreads for fluorescence *in-situ* hybridization on GTG-banded chromosomes were prepared using phytohemagglutinin stimulated blood lymphocytes from a normal male dog. Cells were harvested and slides prepared using standard cytogenetic techniques. Prior to FISH, metaphase chromosome spreads were photographed using a highly sensitive CCD camera and IPLab 2.2.3 (Scanalytics, Inc.). The chromosomes were identified according to the established GTG-banded and DAPI-banded karyotype of the domestic dog (Reimann *et al.* 1996, Breen *et al.* 1999b). FISH on GTG-banded chromosomes was performed using the digoxigenin-labeled BAC DNA. Sonicated total canine DNA and salmon sperm were used as competitors in this experiment. After hybridization overnight, signal detection was performed using a Digoxigenin–FITC Detec-

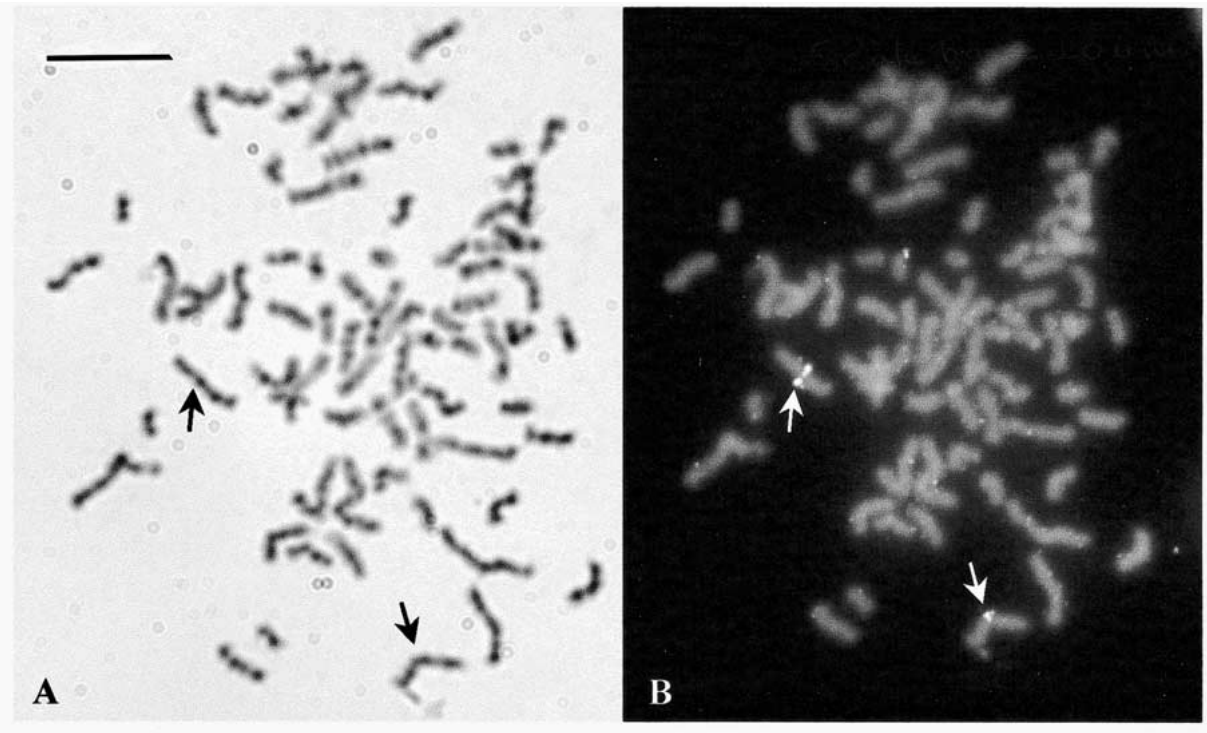


Figure 1. (**A**) Chromosome banding pattern of a canine metaphase spread of a normal male dog obtained by GTG-banding. The arrows indicate chromosome 5. Scale bar = 20 μm. (**B**) Chromosome assignment of the canine *MYO15A* gene by FISH analysis. The digoxigenin labeled clone RPCI81_362O13 containing parts of the canine MYO15A gene was hybridized to metaphase chromosomes. Double signals indicated by arrows are visible on both chromosomes 5q. The chromosomes were counterstained with propidium iodide and DAPI staining.

tion Kit (Quantum Appligene). The chromosomes were counterstained with DAPI and embedded in propidium iodide/antifade. Metaphase spreads were re-examined after hybridization with a Zeiss Axioplan 2 microscope equipped for fluorescence.

Observations and discussion

The canine *MYO15A* BAC hybridized to CFA5q23-q24 (Figure 1B). The position of *MYO15A* on the DAPI-banded CFA5 idiogram is demonstrated in Figure 2.

For fine mapping, the localization of this BAC clone was performed on the canine RHDF5000 radiation hybrid panel (Breen *et al.* 2001). The panel consists of 126 clones, complemented by a canine and hamster genomic DNA as a control. The sequence data for the SP6 BAC end (EMBL AJ428858) were used to design canine-specific primers for RH mapping (5′-TTTCCACATCCACTCTCACG-3′ and 5’-GAAGGGGGAGAAGCAGACTT -3′) and a 201-bp PCR product was obtained only on dog genomic DNA. Two independent PCR reactions were performed in a total of 10 μl using 50 ng of RH cell line DNA. PCR products were separated on 2% agarose gels. Scoring of PCR products was carried out independently by two investigators. The typing data were merged with previously published data and analysis was done using the MultiMap package (Breen *et al.* 2001). The RH results confirmed the chromosomal location obtained by FISH. The two-point analysis revealed that *MYO15A* is linked to the unlinked marker *C02608* with a Lod score of 8.097. This marker *C02608* has been located on the dog genetic linkage map 4.6 cM distal of the solute carrier family 2 member 4 gene (*SLC2A4*) anchored on CFA5 (Breen *et al.* 2001), on a syntenic region of HSA17p, which is in good concordance with the location of the human ortholog *MYO15A* on HSA17p11.2 and *SLC2A4* on HSA17p13 (Figure 2; Thomas *et al.* 2001). In this report we mapped a canine BAC clone containing *MYO15A* to the dog chromosome 5. The assignment of the canine *MYO15A* gene confirms the previously established synteny of HSA17p and CFA5q that was firstly postulated upon the mapping of *SLC2A4* formerly termed glucose transporter 4 gene (*GLUT4*) to CFA5q (Werner *et al.* 1997). The mapping of *MYO15A* on CFA5q23-q24 represents a centromeric enlargement of the homologous chromosome region on HSA17 (Thomas *et al.* 2001) now containing the HSA17p11.2-p13 subbands and refines the comparative map of dog chromosome 5.

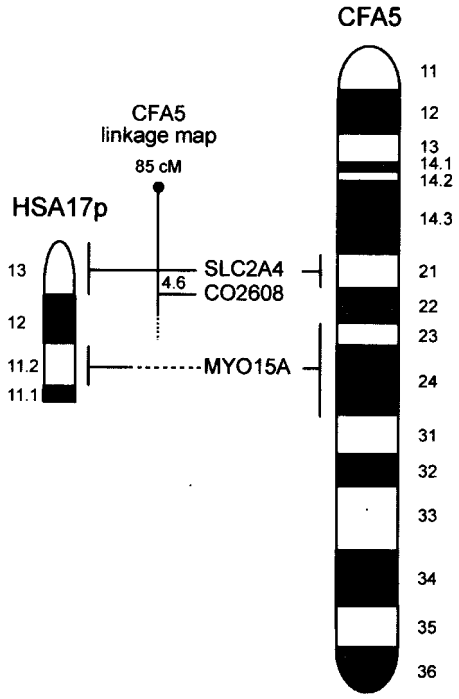


Figure 2. Comparative map of CFA5q21-q24. The position of *MYO15A* on the DAPI-banded CFA5 idiogram is demonstrated and the placement of the linked marker on the canine linkage map was taken from Breen *et al.* (2001). The short arm of HSA17 indicates the homologous human chromosome segment flanked by *SLC2A4* and *MYO15A*.

Acknowledgements

The authors are grateful to Heike Klippert and Stefan Neander for expert technical assistance. This project and Simone G. Rak are supported by a grant from the Gesellschaft zur Förderung Kynologischer Forschung (GKF) e.V., Bonn. Heidi Kuiper is supported by a grant from the affirmative action program for women of the

School of Veterinary Medicine Hannover. Pascale Quignon is supported by funds from the Conseil Regional de Bretagne.

References

Breen M, Thomas R, Binns MM, Carter NP, Langford CF (1999a) Reciprocal chromosome painting reveals detailed regions of conserved synteny between the karyotypes of the domestic dog (*Canis familiaris*) and human. *Genomics* **61**: 145–155.

Breen M, Bullerdiek J, Langford CF (1999b) The DAPI banded karyotype of the domestic dog (*Canis familiaris*) generated using chromosome-specific paint probes. *Chromosome Res* **7**: 401–406.

Breen M, Jouquand S, Renier C *et al.* (2001) Chromosome-specific single-locus FISH probes allow anchorage of an 1800-marker integrated radiation-hybrid/linkage map of the domestic dog genome to all chromosomes. *Genome Res* **11**: 1784–1795.

Johnsson LG, Hawkins JE, Muraski AA, Preston RE (1973) Vascular anatomy and pathology of the cochlea in Dalmatian dogs. In: deLorenzo AJD, ed. *Vascular Disorders and Hearing Defects*. Baltimore: Univ Park Press, pp 249–295.

Mair IWS (1976) Hereditary deafness in the Dalmatian dog. *Arch Otorhinolaryngol* **212**: 1–14.

Probst FJ, Fridell RA, Raphael Y *et al.* (1998) Correction of deafness in shaker-2 mice by an unconventional myosin in a BAC transgene. *Science* **280**: 1403.

Reimann N, Bartnitzke S, Bullerdiek J *et al.* (1996) An extended nomenclature of the canine karyotype. *Cytogenet Cell Genet* **73**: 140–144.

Thomas R, Breen M, Deloukas P, Holmes NG, Binns MM (2001) An integrated cytogenetic, radiation-hybrid, and comparative map of dog chromosome 5. *Mamm Genome* **12**: 371–375.

Wang A, Liang Y, Fridell RA *et al.* (1998) Association of unconventional myosin MYO15 mutations with human nonsyndromic deafness DFNB3. *Science* **280**: 1447–1451.

Werner P, Raducha MG, Prociuk U, Henthorn PS, Patterson DF (1997) Physical and linkage mapping of human chromosome 17 loci to dog chromosomes 9 and 5. *Genomics* **42**: 74–82.

Chapter 6

Assignment of the canine tectorin alpha gene (TECTA) to CFA5q12 →q13 by FISH and confirmation by radiation hybrid mapping

Cord Drögemüller, Simone G. Rak, Heidi Kuiper, Tosso Leeb, Pascale Quignon, Francis Galibert and Ottmar Distl

Published in Cytogenetic and Genome Research 97 (2002) 140A

Cytogenet Genome Res 97:140/A1–3 (2002)
DOI: 10.1159/000064044

Cytogenetic and Genome Research

Assignment[1] of the canine tectorin alpha gene (TECTA) to CFA5q12→q13 by FISH and confirmation by radiation hybrid mapping

C. Drögemüller,[a] S.G. Rak,[a] H. Kuiper,[a] T. Leeb,[a] P. Quignon,[b] F. Galibert,[b] and O. Distl[a]

[a] Institute of Animal Breeding and Genetics, School of Veterinary Medicine Hannover, Hannover (Germany);
[b] UMR 6061 CNRS, Génétique et Développement, Faculté de Médecine, Rennes (France)

[1] To our knowledge this is the first time this gene has been mapped in dogs.

Rationale and significance

The mammalian tectorial membrane is an extracellular matrix of the inner ear which contacts the stereocilia bundles of specialized sensory hair cells. This membrane of the cochlear duct is positioned above these hair cells and provides a mass against which the stereocilia can bend and leads to fluctuations in hair-cell membrane potential, transducing sound into electrical signals (Steel and Kros, 2001). The gelatinous tectorial membrane contains two noncollagenous glycoproteins, alpha- and beta-tectorin (Legan et al., 1997). Mutations in the human tectorin alpha gene (TECTA), which has been mapped to HSA11q22→q24 (Hughes et al., 1998), cause autosomal dominant hearing impairment DFNA8 and DFNA12 in man (Verhoeven et al., 1998). Alpha-tectorin mutations can also be responsible for recessive forms of deafness (DFNB21; Mustapha et al., 1999). Mice homozygous for a targeted deletion in alpha-tectorin have tectorial membranes that are detached from the cochlear epithelium and lack all noncollagenous matrix (Legan et al., 2000). Alpha-tectorin may be involved in the development of hereditary deafness in dogs which has been described in many canine breeds (Strain, 1996, 1999). Several case reports showed congenital deafness in dogs with histological alterations of the tectorial membrane (Branis and Burda, 1985; Hiraide and Paparella, 1988; Coppens et al., 2000). Therefore, the presented mapping of a canine BAC clone containing the TECTA gene to chromosome CFA5q12→q13 by FISH and RH mapping provides the basis for future linkage studies that might clarify the role of TECTA as a putative candidate gene for deafness in dogs.

Materials and methods

Isolation of the canine TECTA gene-containing BAC clone

For the isolation of a canine TECTA-containing genomic DNA clone the comparative approach, i.e. to use appropriate human or murine heterologous screening probes for the identification of gene-specific clones in animals, has been used. The human IMAGE cDNA clone IMAGp998M121786 was provided by the Resource Center/Primary Database of the German Human Genome Project (http://www.rzpd.de/) and sequencing of the 0.8-kb cDNA probe confirmed 100% identity to human tectorin alpha (TECTA) mRNA (GenBank NM_005422). High-density clone filters of the 8.1-fold canine genomic BAC library RPCI-81 (Li et al., 1999) constructed in pBACe 3.6 were screened according to the RPCI protocols (http://www.chori.org/bacpac/). The insert of the human TECTA cDNA clone were labeled with ^{32}P and used as probe. DNA of a positive BAC clone (RPCI81_59C2) was isolated using the Qiagen plasmid maxi kit (Qiagen, Hilden, Germany). BAC DNA termini were sequenced with the ThermoSequenase kit (Amersham-Biosciences, Freiburg, Germany) on a LI-COR 4200L-2 automated sequencer (MWG Biotech, Ebersberg, Germany).

Fluorescence in situ hybridization (FISH) analysis

Canine metaphase spreads for FISH on GTG-banded chromosomes were prepared as described by Breen et al. (1999b). The BAC clone containing the canine TECTA gene was labeled for FISH by nick translation using a Nick-Translation-Mix (Boehringer Mannheim, Germany). Identification of

This project and S.G.R. are supported by a grant of the Gesellschaft zur Förderung Kynologischer Forschung (GKF) e.V., Bonn. H.K. is supported by a grant from the affirmative action program for women of the School of Veterinary Medicine Hannover. P.Q. is supported by funds from the Conseil Regional de Bretagne. The authors are grateful to Heike Klippert and Stefan Neander for expert technical assistance.

Received 31 January 2002; manuscript accepted 18 March 2001.

Corresponding author: Dr. Cord Drögemüller
Institute of Animal Breeding and Genetics
School of Veterinary Medicine Hannover
Bünteweg 17p, D–30559 Hannover (Germany)
telephone: +49-511-9538866; fax: +49-511-9538582
e-mail: cord.droegemueller@tiho-hannover.de

C.D. and S.G.R. contributed equally to the work.

KARGER
Fax + 41 61 306 12 34
E-mail karger@karger.ch
www.karger.com

0301–0171/02/0972–0139$18.50/0

Accessible online at:
www.karger.com/journals/cgr

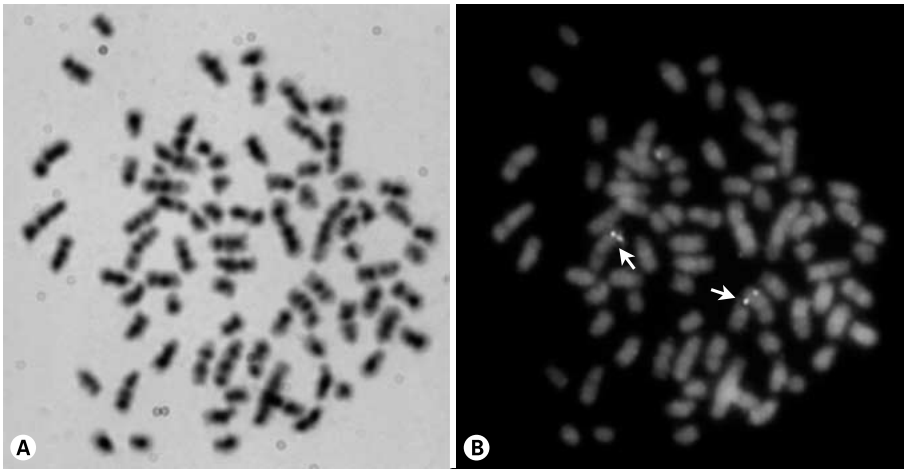


Fig. 1. Chromosome assignment of the canine TECTA gene by FISH. (A) GTG-banding of the canine metaphase spread. (B) Detection of double signals indicated by arrows are visible on both chromosomes 5q.

the chromosomes was done according to the established GTG-banded and DAPI-banded karyotype of the domestic dog (Reimann et al., 1996; Breen et al., 1999a).

Probe name: RPCI81_59C2
Probe type: canine genomic BAC clone
Insert size: 150 kb
Vector: pBACe 3.6
Proof of authenticity: DNA hybridization
Gene reference for the human TECTA gene: Verhoeven et al. (1998)

Radiation hybrid (RH) mapping

A pair of PCR primers for RH mapping (5′-CCGCATAAACATGCTGACAC-3′ and 5′-ACATGGGGTGTTGGTAGGAA-3′) were designed from the generated SP6 sequence of the BAC clone (EMBL AJ428861) and a PCR product of 194 bp was obtained using dog genomic DNA. The marker was mapped on the RHDF5000 panel (Vignaux et al., 1999) according to the described protocols (Priat et al., 1998). After typing the RHDF5000 panel in duplicate, the genotyping data were computed with the MultiMap software (Matise et al., 1994) on the latest radiation hybrid map (Breen et al., 2001).

Results and discussion

The canine BAC clone RPCI81_59C2 was retrieved from the BAC library (Li et al., 1999) using a human cDNA TECTA probe. Sequencing of the canine T7 BAC terminus (EMBL AJ428860) revealed a match of 77% identity over 639 bp (BLAST E-value $< 10^{-57}$) to the end of the 168-kb human genomic DNA chromosome 11q24.1 clone RPP11_709H4 sequence (GenBank AP000826), which contains the human TECTA gene approximately 40 kb downstream (http://www.ensembl.org/).

The chromosomal location of the canine TECTA gene was determined by FISH using the BAC clone to metaphase chromosomes (Fig. 1). This BAC has been assigned to CFA5q12→q13.

Mapping data
Location: 5q12→q13
Number of cells examined: 40
Number of cells with specific signal: 0 (0), 1 (1), 2 (8), 3 (11), 4 (20) chromatids per cell
Most precise assignment: 5q12→q13
Location of background signal (sites with >2 signals): none observed

Moreover, the localization of this BAC clone was performed on the canine RHDF5000 radiation hybrid panel (Vignaux et al., 1999). The STS marker used for RH mapping was designed from the SP6 BAC end sequence. Using the MultiMap package, two-point analysis revealed that TECTA is linked to the marker Ren42N13 situated on CFA5 with a Lod score of 19.67. This chromosome assignment of TECTA to CFA5 is in good agreement with known homology between HSA11q and CFA5q (Thomas et al., 1999). The marker Ren42N13 is linked on the RH map of CFA5 proximal to the Thy-1 cell surface antigen gene (THY1) and the CD3E antigen, epsilon polypeptide (TiT3 complex) gene (CD3E) (Breen et al., 2001). THY1 has been mapped to HSA11q22.3→q23 (Seki et al., 1985) and annotated on HSA11q23.3 (http://www.ensembl.org/). CD3E was physically mapped on HSA11q23 (Gold et al., 1987). Therefore, the order of these genes on CFA5q seems to be conserved with respect to the order of genes on the corresponding segment of HSA11q23→q24.

References

Branis M, Burda H: Inner ear structure in the deaf and normally hearing Dalmatian dog. J Comp Pathol 95:295–299 (1985).

Breen M, Bullerdiek J, Langford CF: The DAPI banded karyotype of the domestic dog *(Canis familiaris)* generated using chromosome-specific paint probes. Chrom Res 7:401–406 (1999a).

Breen M, Langford CF, Carter NP, Holmes NG, Dickens HF, Thomas R, Suter N, Ryder EJ, Pope M, Binns MM: FISH mapping and identification of canine chromosomes. J Hered 90:27–30 (1999b).

Breen M, Jouquand S, Renier C, Mellersh CS, Hitte C, Holmes NG, Cheron A, Suter N, Vignaux F, Bristow AE, Priat C, McCann E, Andre C, Boundy S, Gitsham P, Thomas R, Bridge WL, Spriggs HF, Ryder EJ, Curson A, Sampson J, Ostrander EA, Binns MM, Galibert F: Chromosome-specific single-locus FISH probes allow anchorage of an 1800-marker integrated radiation-hybrid/linkage map of the domestic dog genome to all chromosomes. Genome Res 11:1784–1795 (2001).

Coppens AG, Resibois A, Poncelet L: Bilateral deafness in a Maltese terrier and a great Pyrenean puppy: inner ear morphology. J Comp Pathol 122:223–228 (2000).

Gold DP, van Dongen JJ, Morton CC, Bruns GA, van den Elsen P, Geurts van Kessel AH, Terhorst C: The gene encoding the epsilon subunit of the T3/T-cell receptor complex maps to chromosome 11 in humans and to chromosome 9 in mice. Proc natl Acad Sci, USA 84:1664–1668 (1987).

Hiraide F, Paparella MM: Histopathology of the temporal bones of deaf dogs. Auris Nasus Larynx 15:97–104 (1988).

Hughes DC, Legan PK, Steel KP, Richardson GP: Mapping of the alpha-tectorin gene (TECTA) to mouse chromosome 9 and human chromosome 11: a candidate for human autosomal dominant nonsyndromic deafness. Genomics 48:46–51 (1998).

Legan PK, Rau A, Keen JN, Richardson GP: The mouse tectorins. Modular matrix proteins of the inner ear homologous to components of the sperm-egg adhesion system. J biol Chem 272:8791–8801 (1997).

Legan PK, Lukashkina VA, Goodyear RJ, Kossi M, Russell IJ, Richardson GP: A targeted deletion in alpha-tectorin reveals that the tectorial membrane is required for the gain and timing of cochlear feedback. Neuron 28:273–285 (2000).

Li R, Mignot E, Faraco J, Kadotani H, Cantanese J, Zhao B, Lin X, Hinton L, Ostrander EA, Patterson DF, de Jong PJ: Construction and characterization of an eight-fold redundant dog genomic bacterial artificial chromosome library. Genomics 58:9–19 (1999).

Matise TC, Perlin M, Chakravarti A: Automated construction of genetic linkage maps using an expert system (MultiMap): a human genome linkage map. Nature Genet 6:384–390 (1994).

Mustapha M, Weil D, Chardenoux S, Elias S, El-Zir E, Beckmann JS, Loiselet J, Petit C: An alpha-tectorin gene defect causes a newly identified autosomal recessive form of sensorineural pre-lingual non-syndromic deafness, DFNB21. Hum Mol Genet 8:409–412 (1999).

Priat C, Hitte C, Vignaux F, Renier C, Jiang Z, Jouquand S, Cheron A, Andre C, Galibert F: A whole-genome radiation hybrid map of the dog genome. Genomics 54:361–378 (1998).

Reimann N, Bartnitzke S, Bullerdiek J, Schmitz U, Rogalla P, Nolte I, Ronne M: An extended nomenclature of the canine karyotype. Cytogenet Cell Genet 73:140–144 (1996).

Seki T, Spurr N, Obata F, Goyert S, Goodfellow P, Silver J: The human Thy-1 gene: structure and chromosomal location. Proc natl Acad Sci, USA 82:6657–6661 (1985).

Steel KP, Kros CJ: A genetic approach to understanding auditory function. Nature Genet 27:143–149 (2001).

Strain GM: Aetiology, prevalence and diagnosis of deafness in dogs and cats. Br Vet J 152:17–36 (1996).

Strain GM: Congenital deafness and its recognition. Vet Clin North Am Small Anim Pract 29:895–907 (1999).

Thomas R, Breen M, Langford CF, Binns MM: Zoo-FISH analysis of dog chromosome 5: identification of conserved synteny with human and cat chromosomes. Cytogenet Cell Genet 87:4–10 (1999).

Verhoeven K, Van Laer L, Kirschhofer K, Legan PK, Hughes DC, Schatteman I, Verstreken M, Van Hauwe P, Coucke P, Chen A, Smith RJ, Somers T, Offeciers FE, Van de Heyning P, Richardson GP, Wachtler F, Kimberling WJ, Willems PJ, Govaerts PJ, Van Camp G: Mutations in the human alpha-tectorin gene cause autosomal dominant non-syndromic hearing impairment. Nature Genet 19:60–62 (1998).

Vignaux F, Hitte C, Priat C, Chuat JC, Andre C, Galibert F: Construction and optimization of a dog whole-genome radiation hybrid panel. Mammal Genome 10:888–894 (1999).

Chapter 7

Assignment of the canine cadherin related 23 gene (CDH23) to chromosome 4q12→q13 by fluorescence in situ hybridization and radiation hybrid mapping

Heidi Kuiper, Simone G. Rak, Cord Drögemüller, Tosso Leeb, Pascale Quignon, Francis Galibert and Ottmar Distl

Published in Cytogenetic and Genome Research 97 (2002) 140B

Cytogenet Genome Res 97:140/B1–2 (2002)
DOI: 10.1159/000064045

Cytogenetic and Genome Research

Assignment[1] of the canine cadherin related 23 gene (CDH23) to chromosome 4q12→q13 by fluorescence in situ hybridization and radiation hybrid mapping

H. Kuiper,[a] S.G. Rak,[a] C. Drögemüller,[a] T. Leeb,[a] P. Quignon,[b] F. Galibert,[b] and O. Distl[a]

[a] Institute of Animal Breeding and Genetics, School of Veterinary Medicine Hannover, Hannover (Germany);
[b] UMR 6061 CNRS, Génétique et Développement, Faculté de Médecine, Rennes (France)

[1] To our knowledge this is the first time this gene has been mapped in dogs.

Rationale and significance

Cadherin related gene (CDH23), which encodes otocadherin is a novel member of the cadherin gene superfamily. CDH23 mutations were found in families with nonsyndromic autosomal recessive deafness (DFNB12), and in families with deafness associated with vestibular dysfunction and retinitis pigmentosa (USH1D), respectively (Bolz et al., 2001; Bork et al., 2001; Petit, 2001). DFNB12 has been located on HSA10q21→q22 by linkage mapping (Chaib et al., 1996). In the waltzer mouse it was shown that mutations in the orthologous *Cdh23* gene cause disorganization of inner ear stereocilia leading to deafness (Di Palma et al., 2001). Cdh23 expression was detected in the murine neurosensory epithelium. Otocadherin is a critical component of hair bundle formation and in *Cdh23* deficient mice the stereocilia organization was disrupted during early hair cell differentiation (Di Palma et al., 2001). Congenital sensorineural deafness in dogs may result from degeneration of the hair cells of the organ of Corti (Strain, 1996; Coppens et al., 2000; Poncelet et al., 2000). Therefore, CDH23 might be a suitable candidate gene for congenital nonsyndromic hearing loss in dogs. To facilitate future linkage studies we report here the assignment of the canine CDH23 gene to CFA4q12→q13 by FISH and RH mapping.

Materials and methods

Isolation and characterization of the CDH23 clone

A genomic DNA clone (RPCI81_99C20) of approximately 150 kb from the canine RPCI-81 BAC library (Li et al., 1999) was isolated after screening high density BAC filters according to the RPCI protocols (http://www.chori.org/bacpac/) with a ^{32}P-labelled insert of the IMAGE cDNA clone IMAGp998A186224, containing the human CDH23 mRNA, provided by the Ressource Center/Primary Database of the German Human Genome Project (http://www.rzpd.de/). DNA of the positive BAC clone was isolated using the Qiagen plasmid maxi kit (Qiagen, Hilden, Germany). BAC DNA termini were sequenced with the ThermoSequenase kit (AmershamBiosciences, Freiburg, Germany) on a LI-COR 4200L-2 automated sequencer (MWG Biotech, Ebersberg, Germany).

Fluorescence in situ hybridization (FISH) analysis

Canine metaphase spreads for FISH on GTG-banded chromosomes were prepared as described by Breen et al. (1999c). The BAC clone containing the canine CDH23 gene was labeled by nick translation using a Nick-Translation-Mix (Boehringer Mannheim, Germany). Identification of the chromosomes was done according to the established GTG-banded and DAPI-banded karyotype of the domestic dog (Reimann et al., 1996; Breen et al., 1999a).

Probe name: RPCI81_99C20
Probe type: canine genomic BAC clone
Insert size: 150 kb
Vector: pBACe 3.6
Proof of authenticity: DNA hybridization
Gene reference of human CDH23: Bolz et al. (2001)

Radiation hybrid (RH) mapping

A pair of PCR primers for RH mapping (5′-TGGACTCTGGTTCCTTCCAG-3′ and 5′-CGGTCAGGCCTCTGAGTAAC-3′) were designed from the SP6 sequence of the BAC clone and this 202-bp STS marker was typed on the RHDF5000 dog/hamster radiation hybrid panel (Vignaux et al., 1999). Amplification reaction was performed as previously described (Priat et al., 1998). The typing data, obtained in duplicate, were incorporated into the latest radiation hybrid map (Breen et al., 2001), using the MultiMap package (Matise et al., 1994).

This project and S.G.R. are supported by a grant of the Gesellschaft zur Förderung Kynologischer Forschung (GKF) e.V., Bonn. H.K. is supported by a grant from the affirmative action program for women of the School of Veterinary Medicine Hannover. P.Q. is supported by funds from the Conseil Regional de Bretagne. The authors are grateful to Julia Achilles for expert technical assistance.

Received 31 January 2002; manuscript accepted 18 March 2002.

Corresponding author: Dr. Cord Drögemüller
Institute of Animal Breeding and Genetics
School of Veterinary Medicine Hannover
Bünteweg 17p, D–30559 Hannover (Germany)
telephone: +49-511-953 8866; fax: +49-511-953 8582
e-mail: cord.droegemueller@tiho-hannover.de

H.K. and S.G.R. contributed equally to the work.

KARGER
Fax + 41 61 306 12 34
E-mail karger@karger.ch
www.karger.com

0301–0171/02/0972–0142$18.50/0

Accessible online at:
www.karger.com/journals/cgr

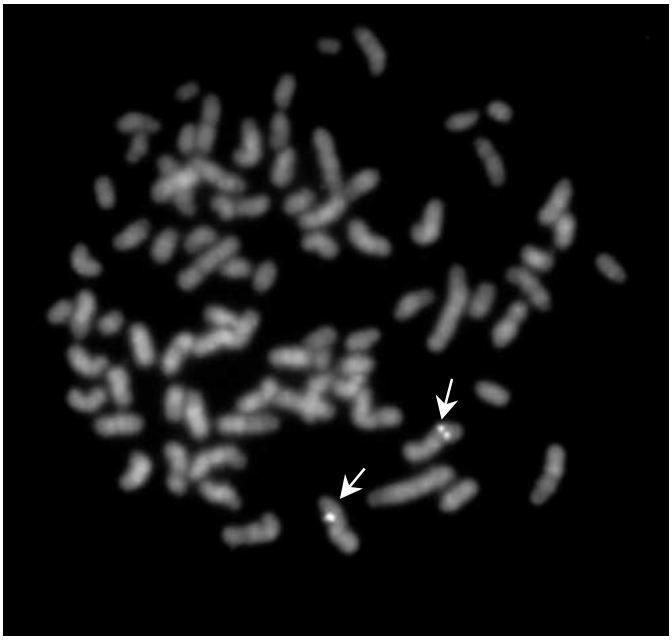


Fig. 1. Chromosome assignment of the canine CDH23 gene by FISH analysis of a canine metaphase spread. The digoxigenin labeled clone RPCI81_99C20 containing parts of the canine CDH23 gene was hybridized to metaphase chromosomes of a normal male dog. Double signals indicated by arrows are visible on both chromosomes 4q. The chromosomes were counterstained with propidium iodide and subsequently identified by DAPI staining.

Results and discussion

The canine BAC clone RPCI81_99C20 was retrieved from the BAC library (Li et al., 1999) using a human cDNA CDH23 probe. Sequencing of the BAC termini revealed a significant match of 95% identity over 76 bp between the SP6 BAC end sequence (EMBL acc. no. AJ428859) and the CDH23 exon 2 sequence (GenBank acc. no. AC016823) which is annotated to genomic DNA HSA10q22.1 clone RP11_472K8 (http://www.ensembl.org/). The chromosomal location of the canine CDH23 gene was determined by FISH analysis using the BAC clone hybridized to metaphase chromosomes (Fig. 1). This BAC clone has been assigned to CFA4q12→q13.

Mapping data:
Location: 4q12→q13
Number of cells examined: 40
Number of cells with specific signal: 0 (1), 1 (2), 2 (6), 3 (5), 4 (26) chromatids per cell
Most precise location: 4q12→q13
Location of background signal (sites with >2 signals): none observed

Moreover this BAC clone was localized on the canine RHDF5000 radiation hybrid panel (Vignaux et al., 1999). The marker used for RH mapping was designed from the SP6 BAC end sequence. Using the MultiMap package, two-point analysis revealed that CDH23 is linked to the marker AHT120 situated on CFA4 with a Lod score of 9.7. The human ortholog is located on HSA10q22.1 (http://www.ensembl.org/), which corresponds well with the synteny data of the canine RH map (Breen et al., 2001). Therefore, CDH23 is the first mapped gene supporting the homology with HSA10 and otherwise confirmed by heterologous FISH painting (Breen et al., 1999b; Yang et al., 1999).

References

Bolz H, von Brederlow B, Ramirez A, Bryda EC, Kutsche K, Nothwang HG, Seeliger M, del C-Salcedo Cabrera M, Vila MC, Molina OP, Gal A, Kubisch C: Mutation of CDH23, encoding a new member of the cadherin gene family, causes Usher syndrome type 1D. Nature Genet 27:108–112 (2001).

Bork JM, Peters LM, Riazuddin S, Bernstein SL, Ahmed ZM, Ness SL, Polomeno R, Ramesh A, Schloss M, Srisailpathy CR, Wayne S, Bellman S, Desmukh D, Ahmed Z, Khan SN, Kaloustian VM, Li XC, Lalwani A, Riazuddin S, Bitner-Glindzicz M, Nance WE, Liu XZ, Wistow G, Smith RJ, Griffith AJ, Wilcox ER, Friedman TB, Morell RJ: Usher syndrome 1D and nonsyndromic autosomal recessive deafness DFNB12 are caused by allelic mutations of the novel cadherin-like gene CDH23. Am J hum Genet 68:26–37 (2001).

Breen M, Bullerdiek J, Langford CF: The DAPI banded karyotype of the domestic dog *(Canis familiaris)* generated using chromosome-specific paint probes. Chrom Res 7:401–406 (1999a).

Breen M, Thomas R, Binns MM, Carter NP, Langford CF: Reciprocal chromosome painting reveals detailed regions of conserved synteny between the karyotypes of the domestic dog *(Canis familiaris)* and human. Genomics 61:145–155 (1999b).

Breen M, Langford CF, Carter NP, Holmes NG, Dickens HF, Thomas R, Suter N, Ryder EJ, Pope M, Binns MM: FISH mapping and identification of canine chromosomes. J Hered 90:27–30 (1999c).

Breen M, Jouquand S, Renier C, Mellersh CS, Hitte C, Holmes NG, Cheron A, Suter N, Vignaux F, Bristow AE, Priat C, McCann E, Andre C, Boundy S, Gitsham P, Thomas R, Bridge WL, Spriggs HF, Ryder EJ, Curson A, Sampson J, Ostrander EA, Binns MM, Galibert F: Chromosome-specific single-locus FISH probes allow anchorage of an 1800-marker integrated radiation-hybrid/linkage map of the domestic dog genome to all chromosomes. Genome Res 11:1784–1795 (2001).

Chaib H, Place C, Salem N, Dode C, Chardenoux S, Weissenbach J, el Zir E, Loiselet J, Petit C: Mapping of DFNB12, a gene for a non-syndromal autosomal recessive deafness, to chromosome 10q21→q22. Hum molec Genet 5:1061–1064 (1996).

Coppens AG, Resibois A, Poncelet L: Bilateral deafness in a maltese terrier and a great pyrenean puppy: inner ear morphology. J Comp Pathol 122:223–228 (2000).

Di Palma F, Holme RH, Bryda EC, Belyantseva IA, Pellegrino R, Kachar B, Steel KP, Noben-Trauth K: Mutations in Cdh23, encoding a new type of cadherin, cause stereocilia disorganization in waltzer, the mouse model for Usher syndrome type 1D. Nature Genet 27:103–107 (2001).

Li R, Mignot E, Faraco J, Kadotani H, Cantanese J, Zhao B, Lin X, Hinton L, Ostrander EA, Patterson DF, de Jong PJ: Construction and characterization of an eight-fold redundant dog genomic bacterial artificial chromosome library. Genomics 58:9–19 (1999).

Matise TC, Perlin M, Chakravarti A: Automated construction of genetic linkage maps using an expert system (MultiMap): a human genome linkage map. Nature Genet 6:384–390 (1994).

Petit C: USHER SYNDROME: From Genetics to Pathogenesis. A Rev Genomics Hum Genet 2:271–297 (2001).

Poncelet L, Coppens A, Deltenre P: Brainstem auditory evoked potential wave V latency-intensity function in normal Dalmatian and Beagle puppies. J Vet Intern Med 14:424–428 (2000).

Priat C, Hitte C, Vignaux F, Renier C, Jiang Z, Jouquand S, Cheron A, Andre C, Galibert F: A whole-genome radiation hybrid map of the dog genome. Genomics 54:361–378 (1998).

Reimann N, Bartnitzke S, Bullerdiek J, Schmitz U, Rogalla P, Nolte I, Ronne M: An extended nomenclature of the canine karyotype. Cytogenet Cell Genet 73:140–144 (1996).

Strain GM: Aetiology, prevalence and diagnosis of deafness in dogs and cats. Br Vet J 152:17–36 (1996).

Vignaux F, Hitte C, Priat C, Chuat JC, Andre C, Galibert F: Construction and optimization of a dog whole-genome radiation hybrid panel. Mammal Genome 10:888–894 (1999).

Yang F, O'Brien PC, Milne BS, Graphodatsky AS, Solanky N, Trifonov V, Rens W, Sargan D, Ferguson-Smith MA: A complete comparative chromosome map for the dog, red fox, and human and its integration with canine genetic maps. Genomics 62:189–202 (1999).

Chapter 8

Development of new gene-associated markers and their linkage with congenital sensorineural deafness in German Dalmatian dogs

Simone G. Rak and Ottmar Distl

Development of new gene-associated markers and their linkage with congenital sensorineural deafness in German Dalmatian dogs

Introduction

Congenital sensorineural deafness (CSD) has been reported to occur in more than 54 different breeds of dogs (Strain, 1996). For almost 100 years, this disorder has been studied most extensively in Dalmatian dogs, since the incidence of CSD in Dalmatians appears to be approximately twice as high as in other affected dog breeds. Different studies demonstrated that beween 16.5 and 29.9% of the Dalmatian dog population exhibit unilateral or bilateral hearing loss (Famula et al., 1996; Holliday et al., 1992; Juraschko et al., 2003a; Muhle et al., 2002; Strain, 2003; Wood and Lakhani, 1997). The most commonly observed histological pattern of degenerative inner ear changes in Dalmatians is known as the cochleo-saccular, or Scheibe, type of end organ degeneration. However, other additional or even deviating alterations have been shown to occur in affected Dalmatians, as well (Anderson et al., 1968; Branis and Burda, 1985; Ferrara and Halnan, 1983; Igarashi et al., 1972; Johnsson et al., 1973; Niparko and Finger, 1997; Mair, 1976; Rouse et al., 1984). Moreover, CSD in Dalmatian dogs seems to be pigment-associated, as previously described for several other dog breeds, as well as for humans, mice, cats, minks and horses (Greibrokk, 1994; Holliday et al., 1992; Juraschko et al., 2003a, 2003b; Mair, 1976; Strain et al., 1992; Strain, 1996). Even though several studies have demonstrated that CSD in Dalmatians is an inherited disease, there are differences in the results of the inheritance mechanism and the number of contributing genes (Anderson, 1968; Famula et al., 1996; Famula et al., 2000; Greibrokk, 1994; Juraschko et al., 2003a; Strain et al., 1992). No gene has yet been identified that is responsible for canine CSD in Dalmatians or in one of the various other dog breeds that suffer from inherited hearing impairment.

Since mutations in various genes have already been found to be the cause of sensorineural hearing impairment in humans or mice, 24 of these genes were considered as candidates for CSD in Dalmatians. A total of 24 different canine BAC clones, each containing at least part of a candidate gene for deafness in Dalmatian dogs, were isolated and mapped to the canine genome by the author and co-workers as previously described (Drögemüller et al., 2002; Kuiper et al. 2002; Rak et al., 2002a, 2002b, 2003).

The objectives of the current study were to develop new, highly polymorphic DNA markers for the 24 candidate genes, including *CDH23*, *CLDN14, COCH, COL11A2, DFNA5, DIAPH1, EDN3, EDNRB, EYA4, GJA1, GJB2, GJB6, MITF, MYH9, MYO6, MYO7A, MYO15A, OTOF, PAX3, POU4F3, SLC26A4, SOX10, TECTA* and *TMPRSS3*, to characterise these markers, and to examine their linkage to CSD in a German Dalmatian dog population.

Material and methods

Pedigree data and DNA isolation

EDTA blood samples were collected from 151 animals from four families segregating for CSD, chiefly composed of large groups of full- and half-sibs. In total, these four families included 169 individuals with an average family size of 42.25 ranging from 13 to 115 animals, and covering two to four generations. The hearing status of all 169 animals was examined by local veterinarians using the BAER (brain stem auditory evoked response) test. The prevalence of CSD in this pedigree was 18.3%; in 16% of the cases the hearing loss was unilateral, in 2.3% bilateral. Amblyacousia was diagnosed in 2.3% of the Dalmatians examined.

The QIAamp® 96 DNA Blood Kit (Qiagen, Hilden, Germany) was used to extract DNA from EDTA blood samples of a subset of 55 Dalmatian dogs including all the affected dogs (unilaterally and bilaterally deaf), their parents and grandparents if available, and, in addition, one to three unaffected animals each from one of four different litters.

Subcloning the BAC DNA

The DNA of each of the 24 candidate gene-containing BAC clones was isolated using the Qiagen plasmid maxi kit (Qiagen, Hilden, Germany). A polymorphic microsatellite marker had already been reported for one isolated and mapped candidate gene (Zemke and Yuzbasiyan-Gurkan, 1999), so that the DNA of only 23 BAC clones was digested with two of the three different enzymes *EcoR* I, *Sac* I or *Xba* I (New England Biolabs, Schwalbach, Germany), and separated on 0.8% agarose gels. The resulting fragments were cloned into the polylinker of pGEM®-4Z (Promega, Mannheim, Germany) and then transformed into XL1-Blue competent *Escherichia coli*. The recombinant plasmid DNA of at least 48 subclones per

BAC clone was isolated using the Montage Plasmid Miniprep$_{96}$ Kit (Millipore, Molsheim, France).

Marker development and genotyping

The generated recombinant plasmid DNA was sequenced using the ThermoSequenase kit (Amersham Biosciences, Freiburg, Germany) on a LI-COR 4200L-2 automated sequencer. Sequence data was analysed using the Sequencher 4.0.5 software (GeneCodes, Ann Arbor, MI, USA), and microsatellites were identified by manual file searching. Before PCR primer designing, repetitive DNA segments such as LINE (long interspersed nuclear element) or SINE (short interspersed nuclear element) elements were identified with the Repeat Masker programme (http://repeatmasker.genome.washington.edu/).

Microsatellite flanking primers for PCR reactions were selected using the Primer3 Input programme (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). Each PCR reaction tube contained 2 µl (~20 ng/µl) genomic dog DNA, 1.2 µl 10x PCR buffer (15 mM $MgCl_2$) (Qbiogene, Heidelberg, Germany), 0.24 µl dimethyl sulfoxide (DMSO) , 0.2 µl dNTPs (100 µM), 0.1 µl (5 U/µl) Taq-polymerase (Qbiogene, Heidelberg, Germany), 0.6 µl (10 µM) 5' IRD700 or IRD800 labelled forward primer, 0.6 µl (10 µM) unlabelled reverse primer and 7.06 µl H_2O in a total volume of 12 µl. The PCR reactions were carried out in PTC-100 or PTC-200 MJ Research thermocyclers with the following programme: 4 min at 94 °C, followed by 32 cycles of 30 sec at 94 °C, 30 sec at maximum annealing temperatures (AT) and a final extension of 45 sec at 72 °C. Primer sequences, their respective AT and repeat type of each microsatellite are listed in Table 1.

For gel analysis, 1 µl of PCR reaction product was mixed with 10-40 µl of formamide loading dye and then loaded on a denaturing 6% polyacrylamide gel (rotiphorese® Gel 40, Carl Roth, Karlsruhe, Germany). The gelelectrophoresis was performed at 48.5 °C on an LI-COR 4200L-2 automated sequencer. Raw data was genotyped by visual examination and manual input.

Statistical analysis

The genotypes for the microsatellite-based markers in combination with pedigree and phenotype data were analysed using the software Merlin (multipoint engine for rapid likelihood inference, Version 0.9.10, Center for Statistical Genetics, University of Michigan,

MI, USA, 2003) in order to detect significant, nonparametric LOD scores for the co-segregation of the gene-associated marker alleles and hearing status.

The data of the genotypes was additionally computed using the software package SAS (Statistical Analysis System, Version 8.2, SAS Institute Inc., Cary, NC, USA, 2002) to specify the number of alleles of each marker, the allele frequency, the observed (h_O) and expected (h_E) heterozygocity values and the polymorphism information content (PIC).

Results

The marker set

In total, 58 microsatellite markers, at least two markers per candidate gene, were tested on an informative group of 55 Dalmatian dogs, including both the affected and unaffected animals and their ancestors. The set of 58 markers was composed of 50 newly developed markers and 8 markers that are included in the canine RH map, which is available at http://www-recomgen.univ-rennes1.fr/doggy.html, for which the author and co-workers have previously shown a linkage to the aforementioned candidate genes. A significant proportion of the genotyped microsatellites (17.2%) proved to be monomorphic in the dogs assessed, and therefore were non-informative for linkage analysis. Another 8.6% were discarded because they could not be amplified or because it was not possible to detect the genotypes. Thus, 43 markers (including 36 newly developed markers and 7 markers of the RH map) of the previously selected 58 markers were ultimately of use for this study.

For most of the 24 candidate genes, two markers were genotyped, and for two candidates three, but for seven candidate genes it was only possible to type one marker each. The marker set is composed of 33 perfect repeats, two imperfect, six compound-perfect and two compound-imperfect repeats. Although the focus was on tetranucleotide repeats, the majority (67.4%) of the markers developed here were shown to be dinucleotide repeats; 20.9% were tetranucleotide repeats, 4.7% trinucleotide repeats and 2.3% pentanucleotide repeats. In addition one marker (2.3%) was a compound di-tetranucleotide and another (2.3%) was a compound tetra-pentanucleotide repeat. Table 1 lists these markers with their respective origin, repeat type, PCR primers and conditions.

In order to determine the utility of these repeats as genetic markers, allele frequencies, h_O, h_E, and PIC values were calculated. The characteristics of each marker are shown in Appendix 1.

These characteristics are summarised for the whole marker set in Table 2, and the allele frequencies are shown in Table 3. The average number of alleles was 3.5 with a minimum of 2 and a maximum of 8 different alleles per marker. The average h_O was 51% with a standard deviation of 19.9%. The worst marker for this characteristic was shown to have a value of 4.4%; the best marker had a h_O value of 84.8%. The h_E value was between 4.3% and 77.7% with an average of 50.3%. The mean PIC value in the genotyped purebred Dalmatian population was 0.49.

Table 2. Characteristics of the developed marker set. The mean values are shown with their respective minimums, maximums, and standard deviations (sd).

feature	min	max	mean	sd
no. of alleles	2	8	3.5	1.7
h_O	4.4	84.8	51.0	19.9
h_E	4.3	77.7	50.3	17.6
PIC	4.3	74.3	48.5	17.3

Table 3. Allele frequencies of the developed set of markers.

no. of alleles	frequency	per cent
2	14	32.56
3	15	34.88
4	4	9.3
5	4	9.3
6	2	4.65
7	3	6.98
8	1	2.32

Table 1. Development of candidate gene associated markers for canine deafness. For each gene the microsatellite-based marker, the marker origin, repeat type, PCR primers and optimised conditions are shown.

Candidate gene	Marker origin	Marker name	Repeat	Primer sequences (5'→3')	AT (°C)	PCR product (bp)
CDH23	RPCI81-99C20	CDH23_MS1	(TTTA)15	F: AATCAGGAGGGGTGAGTGTG R: CCCCCAGCTCATACATTCTC	62	175
	RPCI81-99C20	CDH23_MS2_F2	(ACC)9(ATC)3	F: CCTGTACTGAATGCTTGAGG R: CATCTCTAGAAGAAGCCTCC	60	109
CLDN14	RPCI81-97L17	CLDN14_MS1	(ATTT)7(GTTT)5	F: TCACATAGCATTATATATGGAC R: TTGAGATGGCTCTTACTGAG	56	123
	RPCI81-97L17	CLDN14_MS2	(CA)21	F: TCGATGATGCTTTCTGGTTG R: AGGCTGTGAAATGGATGGAG	60	156
	RPCI81-97L17	CLDN14_MS3	(GA)20	F: GAGAAGCACCAGGCATAGG R: TGGTTTAGCAAGGCTGTTCC	60	146
COCH	RPCI81-321I4	COCH_MS1	(CTTT)~20	F: TGACATACGGAGGACCAAGAG R: CCCCTCCCTTGCTCTATCTC	62	179
	RPCI81-321I4	COCH_MS2	(GA)13	F: TGCCCCTCAGAGATAATCAC R: CTTCAATTATACACACAGGTAC	58	259
COL11A2	RPCI81-24M6	COL11A2_MS1	(AC)20(AG)9	F: TGAATATGGGGCTGAGGAAG R: TTCTCCCTCTGCCTGTGTC	60	151
	RPCI81-24M6	COL11A2_MS3	(CT)16 TT (CT)5	F: GGTTTAGCACTGCCTTCAGC R: CATTAAGCATCTGGCATGTGG	62	241
DFNA5	RPCI81-66C12	DFNA5_MS1	(AAAAT)~25	F: GAAAACTCAGATTAGCCTGG R: ATCTTGAGAGCAAAGGTTGTG	58	219
	RPCI81-66C12	DFNA5_MS2	(CT)11(GT)4(CT)2	F: TGGTTAGGGCATGATTCCAG R: CATGTATAAAGAGTAATGCCAG	58	186
DIAPH1	RPCI81-362I5	DIAPH1_MS1	(ATTT(T))23	F: CGGGAGAGGGTTTGACTAC R: CTCCGTATTGCTCATCTTTCC	60	214
	RPCI81-362I5	DIAPH1_MS2	(AC)22	F: AGCTTCCCTTCTCTGAGAC R: GAGAATAGAGTTTGTGCTCAG	58	191

Table 1. Continued

Candidate gene	Marker origin	Marker name	Repeat	Primer sequences (5'→3')	AT (°C)	PCR product (bp)
EDN3	RPCI81-366E14	EDN3_MS1	(TAGA)12	F: GCTAGGAAAAATCCGCAATG R: GACCCCCTAGGACACCAAC	60	147
EDNRB	Zemke et al.(1999)	EDNRB _MS1	(GA)25	F: GAGAATTGGGCATGGGCAGA R: TGACTTTATCACTGGTCTTTG	58	131
EYA4	RPCI81-301N19	EYA4_MS1	(GT)10(AT)13	F: TTATGCAGCCCATGACAATC R: CAAGGGAACTCAAAGGCTTG	58	258
	RPCI81-301N19	EYA4_MS2	(AG)21	F: TGGACCAGGTCAGTTTGTG R: TCTGCCTGTGTCTCTGCC	58	227
GJA1	RPCI81-370A16	GJA1_MS1	(GT)16	F: ATGGCATGAAGAGGATACCG R: AGGACAGGTGACGGCTCTAC	60	134
	RPCI81-370A16	GJA1_MS2	(AG)12	F: GCTAGTACTCGATTGTGGTC R: TCATGGGTTGTGAGATCCAG	60	190
GJB2	RPCI81-133O22	GJB2_MS1	(CA)12	F: TTAATTTGCTCGTCTTCCTG R: TGTAAGCTCCACGGATCACC	58	148
	RPCI81-133O22	GJB2_MS2	(GA)8	F: CTCTCTTGGTCTCCCTCTGC R: GGGAGTAGGGGTGGAGTAGG	62	195
GJB6	RPCI81-343C15	GJB6_MS2	(CTTT)~20	F: GGTGTTTCCTTTCCTTTTCT R: GGTGTTCTCTCCCTTTCTCT	58	218
	FH2324	GJB2+6_MS1	(GAAA)~25	F: CTCTATGAAAGGTGATTGCC R: CAGCCATACAAATGAGAATTG	58	260
MITF	RPCI81-119P24	MITF _MS2	(GT)12	F: CTACAGTGAATCAGCACAGAC R: CAGCCTTGACTGTTTCTTTGG	62	181
	REN100J13	MITF _MS3	(CA)15	F: TGATTGACTCTACTTTACACA R: TATATTAGGCGGTTTTCTTCT	56	164
MYH9	RPCI81-374A12	MYH9_MS2	(AG)11	F: ACCCAGGTGGCCTGATTG R: GCACGCACGTTCTCTCTTTC	58	96
	FH2293	MYH9_MS3	(GAAA)~43	F: GAATGCCCTTCACCTTGAAA R: GGAAAAGGAGAGATGATGCC	58	227
MYO6	RPCI81-156P14	MYO6_MS2	(AC)13	F: TCTTCCTTGGAAAGGGAACTC R: TGCCCTAACACTTGGAATGG	62	94

Table 1. Continued

Candidate gene	Marker origin	Marker name	Repeat	Primer sequences (5'→3')	AT (°C)	PCR product (bp)
MYO7A	RPCI81-193O2	MYO7A _MS1	(TC)13	F: TGGTTAAAACATTAAACTTATAG R: TAGTATATAGAGATGCAATGG	56	293
	RPCI81-193O2	MYO7A _MS2	(AC)12	F: CATTGGGTGCTTTCCTGTTC R: TGGAGCTGCAGGTATAGCC	60	166
	AHTH298	MYO7A _MS3	(GT)12	F: CCAGGCATTCGAGGGTG R: CAGAACTTGAGGAACCATAG	56	100
MYO15A	RPCI81-362O13	MYO15A _MS1	(AC)18	F: CCATGAACTTTGTGGAACTGC R: AAAGGGTTGCTGTGGAGATG	62	137
	RPCI81-362O13	MYO15A _MS2	(GT)12	F: AGGCAGGTTCATCTGTGTCC R: TCCCAGACCCAGCTACATTC	62	174
OTOF	RPCI81-198L15	OTOF _MS1	(TA)3(TG)9(TA)2(CA)2 C (TAAA)5	F: CAGCCAACTGTATTCTCCTTG R: ATCTTGAGCCCTGCATTAGG	62	197
PAX3	RPCI81-257H23	PAX3_MS1	(AAT)18	F: GAAGCGAGGAGAGACAGTCC R: AAGGAAGCCTCCTGACAACC	60	164
	RPCI81-257H23	PAX3_MS2	(CT)13	F: CAGGGTCAGGCTCTATGCTC R: TCCTATCATCCGGCTTTGAC	60	201
POU4F3	G2C02.466	POU4F3_MS4	(TG)13	F: TCTGGATTGTGGTCACAACC R: ACTGGACACTTCTTTTCAGACG	58	160
SLC26A4	RPCI81-47P17	SLC26A4_MS2	(CT)17	F: AAAGTGGCTGGTTCGGAAG R: AGCAGCAGCATACATTCCTC	58	297
SOX10	RPCI81-505H2	SOX10_MS2	(TAAA)14	F: AAGTAGATCCTATTATCGTGG R: AGTTTCAGTGTCTGTTAAATAG	56	267
TECTA	RPCI81-59C2	TECTA _MS1	(GT)3 CC (GT)19	F: CCGGATTTCTGAGGAGGC R: CATGCTCTTCACCAGAACC	58	140
	RPCI81-59C2	TECTA _MS2	(ATTT)12	F: TCAGCATGGATTTTGTAAAATC R: GGACTGCGTGGACATCTG	58	276
TMPRSS3	RPCI81-125P17	TMPRSS3_MS1	(TC)8(CA)5(CG)2(CA)9	F: ACACGGTTCTCGCTGATGTG R: TGAAGGGGATTGAACAGAGG	62	228
	AHTH246	TMPRSS3_MS2	(GT)16	F: TTCATTCCGAGGTTCTAACTG R: CACCATCTCGTAGCCTTTATC	60	260

Linkage analysis

The evaluation of the data using the Merlin software was based on a multipoint analysis of the pedigree data, including IBD (identical by descent) calculations, kinship calculations and nonparametric linkage (NPL) analysis. This method ignored unaffected animals and looked for alleles that were shared by affected individuals. Moreover, Merlin estimated haplotypes by finding the most likely path of gene flow (Abecasis et al., 2002). In the present study no distinction was made between unilaterally and bilaterally deaf dogs. While the All mode tested the association of all genotyped markers with the phenotype, the Pairs mode tested in pairs the alleles of the markers with the phenotypic expression of the disease. With both methods, a LOD score was used in consideration of the linear model of Kong and Cox (1997). Appendix 2 lists the calculated statistical values for each marker. A significant co-segregation (p (probability) < 0.05) of a marker allele and the phenotypic expression of deafness in the German Dalmatian population was detected for five markers that were developed for the candidate genes *Gene2* and *COL11A2*. The p values reached 0.2 for another four markers, which belong to *GJB2* and *GJB6* (Table 4). Considering the number of genotyped animals it cannot be completely precluded that these two genes are associated with deafness, despite the fact that their p values were not significant.

Table 4. Zmeans and LOD scores with their respective error probabilities for the gene-associated markers of the candidate genes *CLDN14*, *COL11A2*, *GJB2* and *GJB6*.

Marker	**All**			
	Zmean	**p_{Zmean}**	**LOD**	**p_{LOD}**
CLDN14_MS1	1.00	0.2	0.63	0.04
CLDN14_MS2	1.02	0.2	0.64	0.04
CLDN14_MS3	1.02	0.2	0.64	0.04
COL11A2_MS1	1.41	0.08	0.85	0.02
COL11A2_MS3	1.31	0.1	0.78	0.03
GJB2_MS1	0.55	0.3	0.14	0.2
GJB2_MS2	0.55	0.3	0.14	0.2
GJB6_MS1	0.55	0.3	0.14	0.2
GJB6_MS2	0.56	0.3	0.15	0.2

Figures 1 and 2 give the haplotypes of the four genotyped families for the markers developed for *CLDN14* and *COL11A2*.

Discussion

In the current study we report the identification of 36 new microsatellite markers associated with candidate genes for CSD in Dalmatian dogs. To our knowledge this is the first study that investigates the linkage between the trait CSD and the large set of candidate genes including *CDH23*, *CLDN14*, *COCH*, *COL11A2*, *DFNA5*, *DIAPH1*, *EDN3*, *EDNRB*, *EYA4*, *GJA1*, *GJB2*, *GJB6*, *MITF*, *MYH9*, *MYO6*, *MYO7A*, *MYO15A*, *OTOF*, *PAX3*, *POU4F3*, *SLC26A4*, *SOX10*, *TECTA* and *TMPRSS3*. In agreement with Brenig et al. (2003) we detected no significant linkage of the *PAX3*-associated markers with CSD.

The marker set

As has been demonstrated in humans (Weber, 1990) it is also known for dog markers that the informativeness of dog markers increases with increasing average number of repeats (Ostrander et al., 1993) and that tetranucleotide repeats have a higher frequency of mutation events in dogs than di- or trinucleotide repeats (Francisco et al., 1996). In the light of these findings and the studies of Werner et al. (1996) and Zajc et al. (1997), both of whom showed that variation within breeds is likely to be reduced, we chose chiefly to type markers with more than ten repeats and, if possible, to type tetranucleotide repeats, as they were most likely to be informative. The 17.2% of the markers that were shown to be monomorphic were usually, but not always, dinucleotide repeats with no more than twelve repeats.

The mean PIC value of 0.49 in the genotyped purebred Dalmatian population was similar to the value of 0.5 calculated for mixed dog breeds by Zajc et al. (1997), but lower than the value of 0.52 detected for the dog population (35 mixed-breed dogs and 15 unrelated beagles) typed by Ostrander et al. (1993). Therefore, this study confirms the results of Werner et al. (1996) and Zajc et al. (1997) as to the reduced variation in purebred dog populations.

Figure 1. Genotypes of Families 1-4 concerning the *CLDN14*-associated markers.

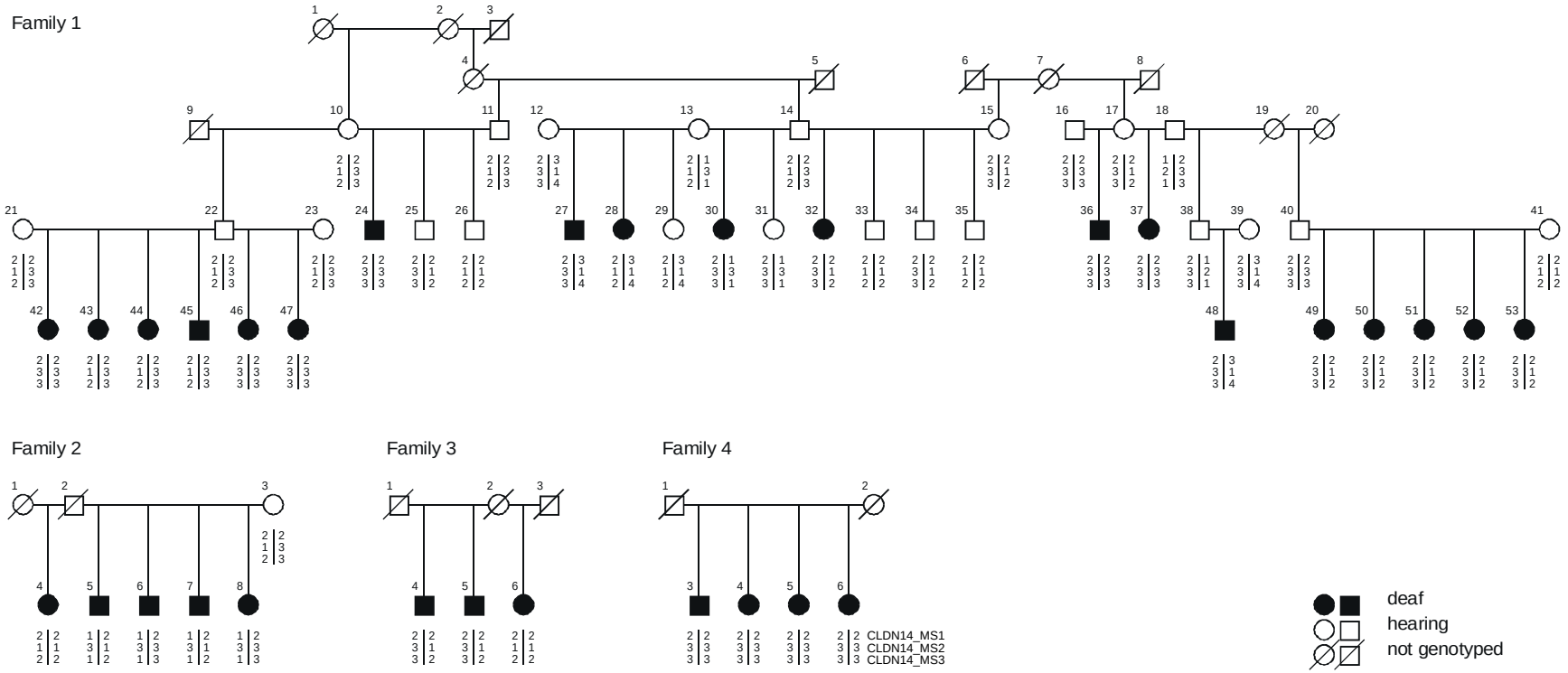
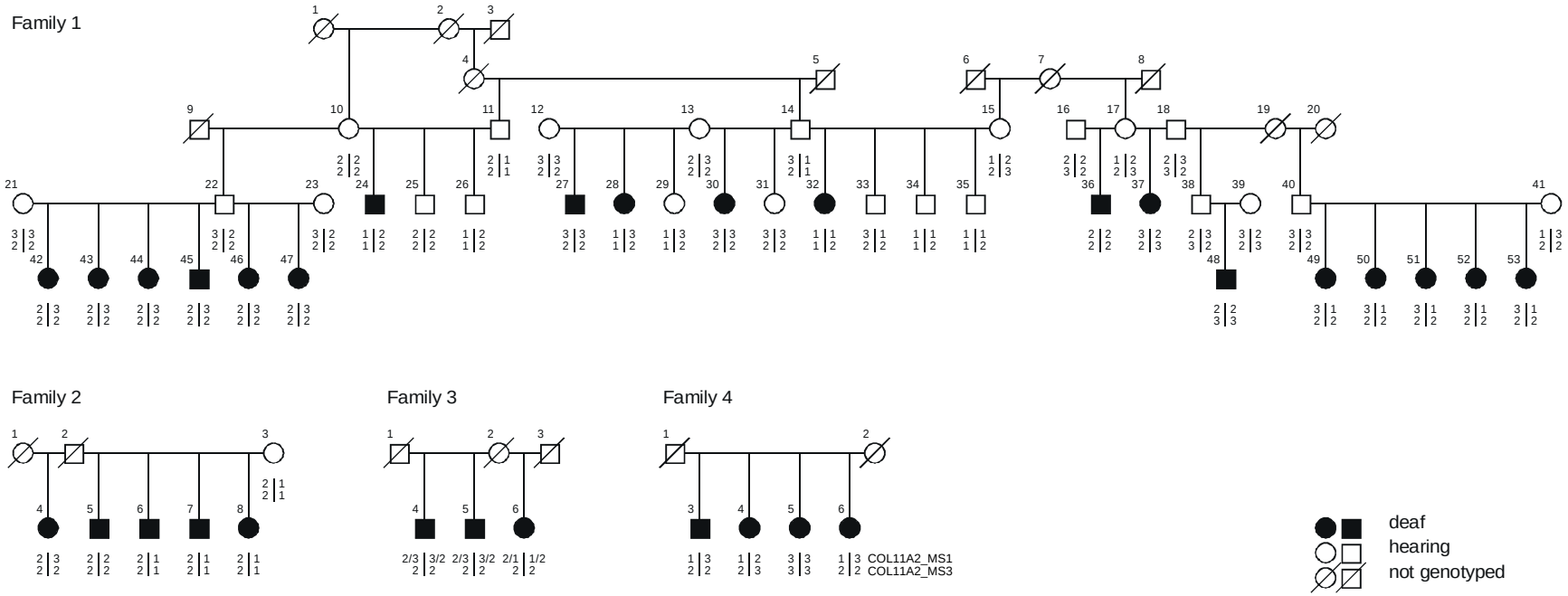


Figure 2. Genotypes of Families 1-4 concerning the *COL11A2*-associated markers.



Due to these very minor discrepancies the developed marker set seemed to be useful for the present study. The majority of the markers were shown to be moderately (PIC 25%-50%) to highly (PIC > 50%) informative, although five markers were only weakly informative with a PIC < 25%. However, for only one candidate gene (*Gene13*) it was not possible to find a marker with a PIC higher than 23%. Therefore it is recommended that the development of additional markers for this gene should be attempted. Contrary to Ostrander et al. (1993), we did not find that markers composed of twelve or fewer repeats always had low PIC values, although we did confirm their findings that the informativeness of genotyped markers increased with increasing repeat number. Furthermore, we also confirmed that markers composed of tetranucleotide repeats usually seem to be informative.

Linkage analysis

Considering a recessive mode of inheritance the genotypes for the *CLDN14*-associated markers presented in Figure 1 confirm the phenotypes detected for the offspring of Dam 17 and Sires 11 and 18 of Family 1, as well as for Family 3. The calculated haplotypes of Family 4 are plausible but they are also less informative than those of the aforementioned animals, because all offspring are homozygous, and none of the parents was available for genotyping. The deaf progeny of Sire 22 in Family 1 and of Sire 2 in Family 2 do not share the same alleles. However, the low information content of Family 4 and the inconsistency of the last mentioned litters may explain why the error probability for *CLDN14* is no lower than 0.04.

For the *COL11A2*-associated markers, the observed genotype correlates to the hearing status in subgroups of Family 1 and in Family 2, with exception of animal 5 in Family 2 (Figure 2). All deaf offspring of Dam 10 in Family 1 have the same haplotype (2,2), and the gene flow is traceable. Moreover, if it is suspected that the haplotype (2,2) of the unaffected dog 26 is exactly the opposite, i.e. the not deafness-causing, allele combination from Dam 10, then all the phenotypes of this subgroup are plausible. The gene flow of Sire 18 to his deaf offspring is not always consistent between the genotyped litters, but within the litters all affected animals share the same alleles. However, Family 3 was shown to be non-informative for *COL11A2* because neither parent was genotyped, and all sibs were homozygous for the marker COL11A2_MS3; consequently it was not possible to calculate the correct haplotypes based only on COL11A2_MS1 (Figure 2).

For *CLDN14* and for *COL11A2* there is no obvious genotype-phenotype interrelation for the half-sib offspring of Sire 14 in Family 1. In this subgroup, some of the deaf and hearing animals have received the same haplotypes of their common parent. For animals 28, 31 and 34, a possible explanation could be an incorrectly determined phenotype or mislabelled blood samples. But in the case of *COL11A2* this would not explain why the deaf animals do not share the same haplotype transmitted by their common parent. Although the chance of recombination was minimised by the development of gene-flanking markers, this possibility must not be totally excluded. Moreover, the haplotypes calculated in the four Dalmatian families seem to indicate that different genes can be responsible for the same deafness phenotype even in related families, as has been demonstrated for human deafness by Adato et al. (2000). One must also take into consideration the way in which these two genes and perhaps other genes not studied here interact for the development of the hearing loss.

Conclusions

In the present study a significant linkage was found for the two genes *CLDN14* and *COL11A2* with congenital sensorineural deafness in the German Dalmatian dog population. It is thus highly probable that these two genes are involved in the development of the disease. With the exception of only one subgroup of the genotyped Dalmatian families, the haplotype calculation showed that either one of the identified genes was responsible for the expression of the affected phenotype in individual nucleus families, or that even both *CLDN14* and *COL11A2* were involved in the hearing loss. The large number of affected animals in the pedigrees analysed that showed a plausible genotype-phenotype correlation reflects the significantly low error probability of the markers associated with *CLDN14* and *COL11A2*. The results indicate that the inheritance of CSD in Dalmatians is at least digenic but probably even heterogenic in origin.

In those cases where the genotype-phenotype interrelation is implausible, a completion of the pedigrees to determine the genotypes of all dams and sires would help exclude the possibility of mislabelled blood samples or of incorrectly determined phenotypes. However, most of the necessary DNA samples are from animals that are no longer alive.

The *CLDN14*- and *COL11A2*-associated markers can now be used to perform an indirect genetic test in order to identify among the hearing Dalmatians those dogs that do not carry the

CSD-linked haplotypes and that therefore have the lowest risk of carrying and transmitting the deafness-causing mutations. Complete sequencing of *CLDN14* and *COL11A2* followed by analysis of mutations would provide information about the responsible deafness-causing mutations and the actual involvement of the identified genes in CSD in Dalmatians. At the same time a direct molecular genetic test for deafness could be developed that could be appliable to larger Dalmatian populations.

The marker set developed here offers the possibility of examining the potential involvement of the selected genes in CSD in Dalmatian populations world-wide. Additionally, with this newly developed set of markers it is now even possible to genotype other dog breeds known to suffer from inherited sensorineural hearing impairment, and to identify those candidate genes that significantly contribute to the diseased phenotype.

Acknowledgements

Simone G. Rak is supported by a grant from the Gesellschaft zur Förderung Kynologischer Forschung (GKF) e.V., Bonn, Germany.

References

ABECASIS, G. R., CHERNY, S. S., COOKSON, W. O. & CARDON, L. R. (2002): Merlin--rapid analysis of dense genetic maps using sparse gene flow trees. Nat. Genet. 30, 97-101.

ADATO, A., RASKIN, L., PETIT, C. & BONNE-TAMIR, B. (2000): Deafness heterogeneity in a Druze isolate from the Middle East: novel OTOF and PDS mutations, low prevalence of GJB2 35delG mutation and indication for a new DFNB locus. Eur. J. Hum. Genet. 8, 437-42.

ANDERSON, H., HENRICSON, B., LUNDQUIST, P. G., WEDENBERG, E. & WERSALL, J. (1968): Genetic hearing impairment in the Dalmatian dog. An audiometric, genetic and morphologic study in 53 dogs. Acta Otolaryngol. Suppl. 232, 1-34.

BRANIS, M. & BURDA, H. (1985): Inner ear structure in the deaf and normally hearing Dalmatian dog. J. Comp. Pathol. 95, 295-9.

BRENIG, B., PFEIFFER, I., JAGGY, A., KATHMANN, I., BALZARI, M., GAILLARD, C. & DOLF, G. (2003): Analysis of the 5' region of the canine PAX3 gene and exclusion as a candidate for Dalmatian deafness. Anim. Genet. 34, 47-50.

DRÖGEMÜLLER, C., RAK, S. G., KUIPER, H., LEEB, T., QUIGNON, P., GALIBERT, F. & DISTL, O. (2002): Assignment of the canine tectorin alpha gene (TECTA) to CFA5q12→q13 by FISH and confirmation by radiation hybrid mapping. Cytogenet. Genome Res. 97, 140A.

FAMULA, T. R., OBERBAUER, A. M. & SOUSA C. A. (1996): A threshold model analysis of deafness in Dalmatians. Mamm. Genome 7, 650-3.

FAMULA, T. R. & OBERBAUER, A. M. & SOUSA, C. A. (2000): Complex segregation analysis of deafness in Dalmatians. Am. J. Vet. Res. 61, 550-3.

FERRARA, M. L. & HALNAN, C. R. (1983): Congenital structural brain defects in the deaf Dalmatian. Vet. Rec.112, 344-6.

FRANCISCO, L.V., LANGSTON, A. A., MELLERSH, C. S., NEAL, C. L. & OSTRANDER, E. A. (1996): A class of highly polymorphic tetranucleotide repeats for canine genetic mapping. Mamm. Genome 7, 359-62.

GREIBROKK, T. (1994): Hereditary Deafness in the Dalmatian: Relationship to Eye and Coat Color. J. Am. Anim. Hosp. Assoc. 30, 170-6.

HOLLIDAY, T. A., NELSON, H. J., WILLIAMS, D. C. & WILLITS, N. (1992): Unilateral and bilateral brainstem auditory-evoked response abnormalities in 900 Dalmatian dogs. J. Vet. Intern. Med. 6, 166-74.

IGARASHI, M., ALFORD, B. R., SAITO, R., COHN, A. M. & WATANABE, T. (1972). Inner ear anomalies in dogs. Ann. Otol. Rhinol. Laryngol. 81, 249-55.

JOHNSSON, L. G., HAWKINS, J. E., MURASKI, A. A. & PRESTON, R. E. (1973): Vascular anatomy and pathology of the cochlea in Dalmatian dogs. In Vascular disorders and hearing defects, ed. deLorenzo, A. J. D., pp. 249-95, Baltimore: University Park Press.

JURASCHKO, K., MEYER-LINDENBERG, A., NOLTE, I. & DISTL, O. (2003a): A regressive model analysis of congenital sensorineural deafness in German Dalmatian dogs. Mamm. Genome 14, 547-54.

JURASCHKO, K., MEYER-LINDENBERG, A., NOLTE, I. & DISTL, O. (2003b): Analysis of systematic effects on congenital deafness in German Dalmatian Dogs. Vet. J. 166, 164-9.

KONG, A. & COX, N. J (1997): Allele-sharing models: LOD scores and accurate linkage tests. Am. J. Hum. Genet. 61, 1179-88.

KUIPER, H., RAK, S. G., DRÖGEMÜLLER, C., LEEB, T., QUIGNON, P., GALIBERT, F. & DISTL, O. (2002): Assignment of the canine cadherin related 23 gene (CDH23) to chromosome 4q12→q13 by fluorescence in situ hybridization and radiation hybrid mapping. Cytogenet. Genome Res. 97, 140B.

MAIR, I. W. (1976): Hereditary deafness in the Dalmatian dog. Arch. Otorhinolaryngol. 212, 1-14.

MUHLE, A. C., JAGGY, A., STRICKER, C., STEFFEN, F., DOLF, G., BUSATO, A., KORNBERG, M., MARISCOLI, M., SRENK, P. & GAILLARD, C. (2002): Further contributions to the genetic aspect of congenital sensorineural deafness in Dalmatians. Vet. J. 163, 311-8.

NIPARKO, J. K. & FINGER, P. A. (1997): Cochlear nucleus cell size changes in the Dalmatian: model of congenital deafness. Otolaryngol. Head. Neck. Surg. 117, 229-35.

OSTRANDER, E. A., SPRAGUE, G. F. JR. & RINE, J. (1993): Identification and characterization of dinucleotide repeat (CA)n markers for genetic mapping in dog. Genomics 16, 207-13.

RAK, S. G., DRÖGEMÜLLER, C., KUIPER, H., LEEB, T., QUIGNON, P., ANDRÉ, C. & DISTL, O. (2002a): Comparative mapping of the canine diaphanous homologue 1 (Drosophila) gene (DIAPH1) to CFA2q23-q24.2. Anim. Genet. 33, 389-90.

RAK, S. G., DRÖGEMÜLLER, C., KUIPER, H., LEEB, T., QUIGNON, P., ANDRÉ, C. & DISTL O. (2002b): Cloning and chromosomal localization of MYO15A to chromosome 5 of the dog (Canis familiaris). Chromosome Res. 10, 407-10.

RAK, S. G., DRÖGEMÜLLER, C., LEEB, T., QUIGNON, P., ANDRÉ, C., SCOTT, A., BREEN, M. & DISTL O. (2003): Chromosomal assignment of 20 candidate genes for canine congenital sensorineural deafness by FISH and RH mapping. Cytogenet. Genome Res. 101, 130-35.

ROUSE, R. C., JOHNSSON, L. G., WRIGHT, C. G. & HAWKINS JR., J. E. (1984): Abnormal otoconia and calcification in the labyrinths of deaf Dalmatian dogs. Acta Otolaryngol. 98, 61-71.

STRAIN, G. M. (1996): Aetiology, prevalence and diagnosis of deafness in dogs and cats. Br. Vet. J. 152, 17-36.

STRAIN, G. M. (2003): Deafness prevalence and pigmentation and gender associations in dog breeds at risk. Vet. J. 167, in press. (http://www.lsu.edu/deafness/ VetJDeaf2003.pdf)

STRAIN, G. M., KEARNEY, M. T., GIGNAC, I. J., LEVESQUE, D.C., NELSON, H. J., TEDFORD, B. L. & REMSEN, L. G. (1992): Brainstem auditory-evoked potential assessment of congenital deafness in Dalmatians: associations with phenotypic markers. J. Vet. Intern. Med. 6, 175-82.

WEBER, J. L. (1990): Informativeness of human (dC-dA)n (dG-dT)n polymorphisms. Genomics 7, 524-30.

WERNER, P., ARNOLD, S., SCHELLING, C. & HUBSCHER, U. (1996): Analysis of microsatellite polymorphisms in Bernese mountain dogs and Newfoundlands. Schweiz. Arch. Tierheilkd. 138, 152-6.

WOOD, J. L. N. & LAKHANI, K. H. (1997): Prevalence and Prevention of Deafness in the Dalmatian - Assessing the Effect of Parental Hearing Status and Gender using Ordinary Logistic and Generalized Random Litter Effect Models. Vet J 154, 121-33.

ZAJC, I., MELLERSH, C. S. & SAMPSON, J. (1997): Variability of canine microsatellites within and between different dog breeds. Mamm. Genome 8, 182-5.

ZEMKE, D. & YUZBASIYAN-GURKAN, V. (1999): A single nucleotide polymorphism and a (GA)n microsatellite in intron 6 of the canine endothelin receptor B (EDNRB) gene. Anim. Genet. 30, 390.

Chapter 9

General discussion

General Discussion

A candidate gene analysis for congenital sensorineural deafness in Dalmatian dogs was carried out for the 24 genes *CDH23*, *CLDN14, COCH, COL11A2, DFNA5, DIAPH1, EDN3, EDNRB, EYA4, GJA1, GJB2, GJB6, MITF, MYH9, MYO6, MYO7A, MYO15A, OTOF, PAX3, POU4F3, SLC26A4, SOX10, TECTA* and *TMPRSS3.* These genes are known to be involved either in human sensorineural non-syndromic deafness or in the human Waardenburg syndrome.

The candidate gene approach in combination with the affected-pedigree-member method

In the past, whole genome screens for canine diseases have not always given satisfactory results (Dukes-McEwan and Jackson, 2002), although efforts have been made to develop and multiplex a dense, genome-covering set of 172 microsatellites for genome-wide genetic linkage studies (Cargill et al., 2002; Richman et al., 2001). Of course, one reason is the difficulty of determining the phenotype of many canine diseases. But more often non-informative or inconclusive markers, which were also too far apart from each other, have meant that large proportions of the canine genome have not been studied. This problem has been further exacerbated by the fact that there often is a reduced polymorphism of microsatellites within a canine breed (Werner et al., 1996; Zajc et al., 1997) or in a line within a breed (Altet et al., 2001). Although the BAER test is a reliable method for determining the hearing status, a whole genome screen for CSD in Swiss Dalmatian dogs was not successful (unpublished data). There have been major advances in the canine genome project with the current version of the comprehensive radiation hybrid map (Guyon et al., 2003), but the utility of this new map for whole genome screens has yet to be demonstrated.

Over the past decade it has become increasingly clear how far structural and functional homologies at the gene level extend across even very distantly related species. Much is known about deafness-causing gene mutations in humans and mice, including the fact that the clinical and histopathological findings are often very similar to those of Dalmatian deafness. Therefore these species seemed to be suitable model organisms for a molecular genetic analysis of CSD in Dalmatian dogs. With two exceptions (see *The chromosomal assignment* below) the 24 genes selected cover all the potential candidate genes for deafness in Dalmatians that have thus far been identified as causing human sensorineural non-syndromic

deafness or pigment-associated deafness. Based upon the number of known human deafness-associated loci for which the responsible gene mutations have not yet been detected, it is to be expected that additional potential human candidates for CSD in Dalmatian dogs will become available in future.

The candidate gene approach which usually ignores pedigree information, was combined in this thesis with the method of affected pedigree members for linkage analysis. The development of a specific candidate gene-associated set of markers circumvented the necessity of sequencing and characterising the genomic structure of each of the selected 24 candidate genes. Once a significant linkage can be determined with this method, only the set of linked genes with the required low error probability values has to be considered for further molecular genetic analysis. In contrast, using the current version of the canine map for whole genome screens one must analyse either all potential candidate genes within a linked region of several cM or, if no promising genes are available within this region, one must use a much denser set of markers, narrowing the previously identified region to between 1 and 2 cM, which then must be completely sequenced. Moreover, because a recombination of the developed gene-flanking markers of the respective genes was kept to a minimum in this study, it was possible to perform linkage analysis with only a fraction of the pedigree material needed for whole genome scans.

The chromosomal assignment

The selected genes were mapped physically by fluorescence *in situ* hybridisation (FISH) and genetically by radiation hybrid (RH) mapping. The obtained mapping results can be regarded as valid for a number of reasons. Concordant results have been obtained for all 24 genes with these two independent methods, and all mapping results were in agreement with known synteny data between human and canine chromosomes on the established human–dog comparative map. Since the concordant results could also have been obtained by mapping false positive BAC clones, this risk was initially circumvented by confirmation of the identity of the isolated clones by ECL hybridisation with the inserts of the respective IMAGE cDNA clones. However, the most important evidence is undoubtedly the fact that the DNA sequencing of the canine BAC fragments followed by a BLAST search of these canine sequences revealed significant matches to the corresponding human genes or to the human chromosomal regions flanking the corresponding genes.

In the beginning of this study two additional candidate genes were also selected: *KCNQ4* (potassium voltage-gated channel, KQT-like subfamily, member 4) and *GJB3* (gap junction protein, beta 3, 31kDa [connexin 31]). Both genes belong to gene families whose members show very similar expression patterns, functions and sequence homologies. Such members of gene families have been shown to be very difficult to map by heterologous hybridisation. In the case of the gene *KCNQ4*, deviating synteny data in combination with BLAST matches to another member of this ion channel gene family gave evidence that the wrong BAC clone had been mapped although the correct cDNA clone was used. The BAC end sequences of the isolated canine clone for *GJB3* and the synteny data showed homologies to the corresponding human chromosome 1. But sequencing of additional subclone fragments showed that it was not *GJB3*, which is located on HSA1p, that had been analysed, but a gene with a very similar sequence located on the q arm of the human chromosome 1. Thus, the plausibility of the results was enhanced by the present strategy for candidate gene mapping with critical inspection of the results by using a combination of ECL hybridisation, sequencing of subclone fragments, mapping with independent methods and comparison of synteny data.
The chromosomal assignment of 24 genes to the canine genome also contributes to the development of a high-resolution comparative dog and human map.

The marker set

In comparison to the studies of Zajc et al. (1997) and Ostrander et al. (1993), who calculated PIC values of 0.5 and 0.52 for predominantly mixed-breed dog populations, and due to the fact that variation within breeds is likely to be reduced (Werner et al., 1996; Zajc et al., 1997), the marker set developed here and genotyped on purebred Dalmatians, with an observed PIC value of 0.49, seemed to be sufficiently informative for linkage analysis. The strategy of developing these gene-associated markers minimised the possibility of recombination between markers and genes. However, it was not possible to type a microsatellite-based marker with sufficient information content for one candidate gene. For this reason it cannot be precluded that this gene might be involved in CSD and the development of additional markers is recommended, a strategy that could also be conceivable for those seven candidate genes for which only one informative marker was found.

The 36 newly developed and physically anchored markers provide not only the possibility of performing linkage studies on hearing impairment, but they also contribute to the resources needed for fine mapping of QTL.

The set of markers developed here offers the possibility of examining the potential involvement of the selected genes in CSD in Dalmatian populations world-wide. Furthermore, with this newly developed set of markers it is now even possible to genotype other dog breeds known to suffer from inherited sensorineural hearing impairment, and to identify those candidate genes that significantly contribute to the diseased phenotype.

Linkage analysis

A significant co-segregation of a marker allele and the phenotypic expression of deafness in the German Dalmatian population was determined for five markers that had been developed for the candidate genes *CLDN14* and *COL11A2*. For another four markers belonging to *GJB2* and *GJB6*, the error probabilities were 0.2. Considering the number of genotyped animals it cannot be totally precluded that the last two genes are associated with deafness, although their statistical values were not significant. Yet it cannot be ruled out that the other newly developed candidate gene-associated markers might show significant effects on hearing loss in more powerful study designs or in Dalmatian breed lines with different genetic backgrounds. For this reason further evaluation of these markers would be appropriate.

The significant linkage indicated that it is highly probable that at least the two genes *CLDN14* and *COL11A2* are involved in the development of the disease. Moreover, the linkage of two or perhaps even four genes reveals that the inheritance of CSD in Dalmatians is at least digenic and probably even heterogenic. The assumption of a heterogenic basis of this disease is further supported by the fact that for one subgroup of the genotyped families none of the selected genes seemed to explain the phenotypes. For all other genotyped animals the haplotype calculation showed that either one of the identified genes was responsible for the expression of the disease in individual nucleus families, or that *CLDN14* and *COL11A2* were both involved in the hearing loss. As in findings on human deafness (Adato et al., 2000), the present results give rise to the suspicion that different genes can be responsible for the same deafness phenotype, even in related Dalmatian families.

Completion of the pedigrees studied in order to detect the genotypes of all dams and sires would help to exclude the possibility of mislabelled blood samples or of incorrectly

determined phenotypes in those cases in which the genotype-phenotype interrelation was implausible. However, most of the additionally needed DNA samples are from animals that are no longer alive.

The *CLDN14*- and *COL11A2*-associated markers can now be used to perform an indirect genetic test in order to identify among the hearing Dalmatians those dogs that do not carry the CSD-linked haplotypes and that therefore have the lowest risk of carrying and transmitting the deafness-causing mutations. In fact, this indirect genetic test can now be applied to dog breeding programmes and can help to identify genotypes that could be used for the analysis of mutations in the respective genes. Complete sequencing of *CLDN14* and *COL11A2* followed by analysis of mutations would provide information about the responsible deafness-causing mutations and the actual involvement of the identified genes in the disease. At the same time, these findings could be translated into the development of a direct genetic test for CSD in Dalmatians.

References

ADATO, A., RASKIN, L., PETIT, C. & BONNE-TAMIR, B. (2000): Deafness heterogeneity in a Druze isolate from the Middle East: novel OTOF and PDS mutations, low prevalence of GJB2 35delG mutation and indication for a new DFNB locus. Eur. J. Hum. Genet. 8, 437-42.

ALTET, L., FRANCINO, O. & SANCHEZ, A. (2001): Microsatellite polymorphism in closely related dogs. J. Hered. 92, 276-9.

CARGILL, E. J., CLARK, L. A., STEINER, J. M. & MURPHY, K. E (2002): Multiplexing of canine microsatellite markers for whole-genome screens. Genomics 80, 250-3.

GUYON, R., LORENTZEN, T. D., HITTE, C., KIM, L., CADIEU, E., PARKER, H. G., QUIGNON, P., LOWE, J. K., RENIER, C., GELFENBEYN, B., VIGNAUX, F., DEFRANCE, H. B., GLOUX, S., MAHAIRAS, G. G., ANDRÉ, C., GALIBERT, F. & OSTRANDER, E. A. (2003): A 1-Mb resolution radiation hybrid map of the canine genome. Proc. Natl. Acad. Sci. USA 100, 5296-301.

DUKES-MCEWAN, J. & JACKSON, I. J (2002): The promises and problems of linkage analysis by using the current canine genome map. Mamm. Genome 13, 667-72.

OSTRANDER, E. A., SPRAGUE, G. F. JR. & RINE, J. (1993): Identification and characterization of dinucleotide repeat (CA)n markers for genetic mapping in dog. Genomics 16, 207-13.

RICHMAN, M., MELLERSH, C. S., ANDRÉ, C., GALIBERT, F. & OSTRANDER, E. A. (2001): Characterization of a minimal screening set of 172 microsatellite markers for genome-wide screens of the canine genome. J. Biochem. Biophys. Methods 47, 137-49.

WERNER, P., ARNOLD, S., SCHELLING, C. & HUBSCHER, U. (1996): Analysis of microsatellite polymorphisms in Bernese mountain dogs and Newfoundlands. Schweiz. Arch. Tierheilkd. 138, 152-6.

ZAJC, I., MELLERSH, C. S. & SAMPSON, J. (1997): Variability of canine microsatellites within and between different dog breeds. Mamm. Genome 8, 182-5.

Chapter 10

Summary

Summary

Molecular genetic analysis of congenital deafness in Dalmatian dogs

Simone Gerlinde Rak

The objective of the present thesis was to select suitable candidate genes for congenital sensorineural deafness (CSD) in Dalmatian dogs, to map these genes to the canine genome, and to develop a candidate gene-associated marker set. This set of markers was intended for linkage analysis in order to identify among the large set of candidate genes those, for which involvement in the development of the disease is highly probable.

A total of 24 human or murine candidate genes were selected that show an appropriate clinical and histological disease pattern in comparison to deafness in Dalmatians. For each candidate gene, one canine BAC clone was isolated by screening a canine BAC library with candidate gene-specific human or murine cDNA clones. The identity of the clones was confirmed by gene-specific heterologous hybridisation of the isolated canine BAC DNA and subsequent DNA sequencing of BAC fragments. BLAST searches of these canine sequences revealed significant matches to the corresponding human genes or to the human chromosomal regions flanking the corresponding genes. The 24 gene-containing BAC clones were assigned to 16 different canine chromosomes by FISH and RH mapping. Concordant results were obtained with these two independent methods. All assignments observed in this study were in good agreement with known synteny data from the established human-dog comparative map. The generated subclone sequences of the mapped BAC clones were used for the development of a set of new microsatellite-based markers. In total 43 candidate gene-associated markers (including 36 newly developed markers and 7 markers from the current RH map) were used for linkage analysis with CSD in an affected-pedigree-member design for German Dalmatian dogs. Significant linkage between the gene-associated markers and hearing loss was found for two of the 24 candidate genes investigated here.

The observed result indicates that the inheritance of CSD in Dalmatians is at least digenic and probably even heterogenic. Moreover, the calculation of the respective haplotypes gives rise to the suspicion that even in related Dalmatian families mutations in different genes may cause the same deafness phenotype. An indirect genetic test for CSD in Dalmatians can now

be performed using the *CLDN14*- and *COL11A2*-associated markers developed here and this test can be applied to dog breeding programmes. Complete sequencing of the two identified genes followed by analysis of mutations would provide information about the responsible deafness-causing mutations and the actual involvement of these genes in the disease. At the same time, these findings could be translated into the development of a direct genetic test for CSD in Dalmatians.

This new set of markers offers the possibility of examining the potential involvement of the selected genes in CSD in Dalmatian populations world-wide. Furthermore, with this new marker set it is now even possible to genotype other dog breeds known to suffer from inherited sensorineural hearing impairment and to identify those candidate genes that significantly contribute to the diseased phenotype.

Chapter 11

Erweiterte Zusammenfassung

Erweiterte Zusammenfassung

Molekulargenetische Untersuchung der kongenitalen Taubheit beim Dalmatiner

Simone Gerlinde Rak

Einleitung

Die kongenitale sensorineurale Taubheit ist eine Erkrankung, von der viele Säugetierspezies betroffen sind. Beim Hund werden über 54 verschiedene Rassen beschrieben, bei welchen ein kongenitaler Hörverlust gehäuft auftritt. In zahlreichen europäischen und amerikanischen Studien weisen hierbei die Dalmatiner mit 16,5-29,9% die höchste Taubheitsinzidenz auf. Die histologischen Veränderungen im Innenohr werden im allgemeinen als cochleo-sacculär beschrieben und können bei betroffenen Tieren schon direkt nach der Geburt diagnostiziert werden. Die Degenerationen im Innenohr schreiten mit zunehmendem Alter der Tiere fort und führen normalerweise spätestens in einem Alter von etwa vier Monaten zu einem vollständigen, ein- oder beidseitigen Hörverlust. Obwohl für den Dalmatiner die Erblichkeit der Erkrankung wiederholt nachgewiesen werden konnte, wurden für die verschiedenen untersuchten Dalmatinerpopulationen auch sehr viele unterschiedliche Erbgänge in Betracht gezogen. Es ist bisher nicht gelungen, die genaue Anzahl der beteiligten Gene zu bestimmen. Auch war es bislang für keine der betroffenen Hunderassen möglich, ein taubheitsverursachendes Gen zu identifizieren.

Bei deutschen Dalmatinern ergab eine Auswertung mittels komplexer Segregationsanalysen, dass ein Modell mit einem rezessiven Hauptgen und einer polygenen Komponente das Auftreten der kongenitalen Taubheit in der untersuchten Population am besten erklärte. Zusätzlich konnte ein Zusammenhang zu Pigmentierungsgenorten nachgewiesen werden, da Tiere mit blauer Augenfarbe signifikant häufiger von Taubheit betroffen waren und gleichzeitig Tiere mit Plattenzeichnung seltener kongenitalen Hörverlust zeigten.

In Deutschland gelten schon seit längerem ein- oder beidseitige Taubheit sowie blaue Augen als zuchtausschließende Fehler. Das bedeutet, dass alle Tiere, die eventuell in der Zucht eingesetzt werden sollen, zuvor mittels der Methode der akustisch evozierten Potentiale (AEP) auf ihr Hörvermögen untersucht werden müssen. Es hat sich allerdings gezeigt, dass allein diese Zuchtbestimmungen nicht genügen, um die hohe Taubheitsinzidenz beim

Dalmatiner deutlich zu reduzieren. Da es sich für Hundebesitzer meistens als sehr schwierig darstellt, einen tauben Hund zu erziehen und entsprechend zu halten, werden fast alle Welpen mit bilateralem Hörverlust euthanasiert. Aus diesen Gründen scheint es nun dringend notwendig zu sein, die kongenitale sensorineurale Taubheit beim Dalmatiner molekulargenetisch zu untersuchen, um ein molekulargenetisches Testverfahren entwickeln und damit auch Anlageträger identifizieren zu können.

Innerhalb des letzten Jahrzehnts wurden auch zwischen relativ unverwandten Spezies, wie beispielsweise zwischen Mensch und Drosophila, die strukturellen und funktionellen Homologien auf genetischer Ebene zunehmend verdeutlicht. Aus diesem Grund kann bei Mensch und die Maus das Wissen über die schon aufklärten, taubheitsverursachenden Genmutationen verwendet werden, um die kongenitale sensorineurale Taubheit beim Dalmatiner möglichst effizient molekulargenetisch aufzuklären.

Das Ziel dieser Dissertation ist es, geeignete humane oder murine Kandidatengene auszuwählen, diese beim Hund zu kartieren und ein kandidatengenassoziiertes Markerset zu entwickeln. Mit diesem Markerset soll dann eine potenzielle Kopplung der ausgewählten Kandidatengene mit der kongenitalen sensorineuralen Taubheit untersucht werden. Die Kandidatengene mit signifikanter Kopplung zur kongenitalen sensorineuralen Taubheit stehen dann für die weitere molekulargenetische Analyse zur Verfügung, um die kausalen Mutationen zu identifizieren.

Die Ergebnisse dieser Studie sollen der molekulargenetischen Aufklärung der kongenitalen sensorineuralen Taubheit beim Dalmatiner und damit der Entwicklung eines Gentestes dienen.

Material und Methoden

Kandidatengenauswahl

Beim Mensch und bei der Maus wurden bisher 32 Gene identifiziert, die einen ursächlichen Zusammenhang zur sensorineuralen, nicht syndromischen Taubheit aufweisen. 19 dieser Gene wurden aufgrund des vergleichbaren klinischen sowie histologischen Krankheitsbildes als Kandidatengene für die canine Taubheit ausgewählt. Da beim Dalmatiner auch eine Assoziation zu Pigmentierungsgenorten zu bestehen scheint, sollten des weiteren die fünf

Gene, die für das menschliche Waardenburg-Syndrom kausal verantwortlich sind, in der Analyse berücksichtigt werden.

Isolierung von caninen genomischen BAC-Klonen

Für die 24 Kandidatengene wurden aus Internet-Datenbanken humane oder murine cDNA-Sequenzen ausgewählt, für die entsprechende IMAGE-Klone am RZPD Berlin zur Verfügung standen. Mit den ^{32}P radioaktiv markierten Inserts dieser cDNA-Klone wurde, entsprechend der Standard RPCI-Protokolle, die canine genomische BAC-Genbank (RPCI-81) durchmustert. Nach Isolierung der BAC-DNA und Spaltung mit verschiedenen Restriktionsenzymen wurden die BAC-Klone zunächst über eine ECL-Hybridisierung mit den entsprechenden cDNA-Sonden verifiziert. Über eine Pulsfeldgelelektrophorese wurde die Größe der caninen genomischen DNA-Fragmente bestimmt. Die BAC-Klone wurden von beiden Rändern auf einem automatischen Sequenziergerät (LI-COR 4200L-2) ansequenziert. Diese Randsequenzen und Subklonsequenzen wurden mit bestehenden Datenbankeinträgen über die Programme BLAST und Ensembl Genome Server verglichen. Zur Identifizierung repetitiver Elemente (SINE, LINE) wurde das Programm Repeat Masker verwendet.

Radiation Hybrid (RH) Kartierung

Für jedes Kandidatengen wurde ein positiver BAC-Klon ausgewählt. Aus den Randsequenzen dieser Klone wurden Primer entwickelt, die über PCR an den Zelllinien des caninen RH Panels RHDF5000 typisiert und zu bereits kartierten Markern physikalisch angeordnet wurden. Die statistische Auswertung erfolgte mit Hilfe der Software Multimap package über eine Zwei-Punkt-Analyse.

Fluoreszenz-in-situ-*Hybridisierung*

Für die Fluoreszenz-*in-situ*-Hybridisierung auf GTG- und DAPI-gebänderten Chromosomen wurden canine Metaphasenchromosomen Phytohämagglutinin-stimulierter Blutlymphozyten eines gesunden Hundes genutzt. Die Präparation der Metaphasen erfolgte nach zytogenetischen Standardtechniken. Die DNA der BAC-Klone wurden über Nick-Translation mit Digoxigenin-11-dUTP, Spectrum Red dUTP, Spectrum Green dUTP, Spectrum Orange dUTP oder DEAC-dUTP markiert und auf den gebänderten caninen Chromosomen hybridisiert. Als Kompetitor, zum Binden repetitiver Sequenzen der markierten Klone, wurde

gescherte genomische canine DNA und Lachssperma-DNA eingesetzt. Die Signale der hybridisierten Proben wurden mit dem Digoxigenin-FITC Detection Kit detektiert. Die Identifikation der Chromosomen erfolgte entsprechend der etablierten GTG- und DAPI-Bänderung des Hundes.

Subklonierung der BAC-DNA und Markersuche

Die kartierten caninen Klone wurden zur Subklonierung jeweils mit mindestens zwei unterschiedlichen Restriktionsenzymen gespalten, anschließend in den pGEM®-4Z Vektor ligiert und zur Kultivierung in kompetente XL1-blue *E. coli* transformiert. Von mindestens 48 Subklonen je Kandidatengen wurde dann die Plasmid-DNA isoliert und von beiden Rändern ansequenziert. Um Mikrosatelliten identifizieren und flankierende Primer entwickeln zu können, wurden diese jeweils etwa 700 bp umfassenden Sequenzen mit den Programmen Sequencher 4.0.5, Repeat Masker, und Primer3 Input bearbeitet.

Genotypisierung

Über PCR und Polyacrylamidgelelektrophorese wurden je Kandidatengen mindestens zwei neuentwickelte, möglichst polymorphe, genassoziierte Mikrosatellitenmarker an für Taubheit segregierenden Dalmatinerfamilien typisiert. Zusätzlich wurden einige, zu den Kandidatengenen gekoppelte Marker für die Genotypisierung verwendet. Die Daten wurden mit dem Softwarepaket SAS (Statistical Analysis System, Version 8.2) bearbeitet, um das Markerset bezüglich Allelfrequenzen, beobachteten (H_O) und erwarteten (H_E) Heterozygotiegrad und PIC (Polymorphism information content) charakterisieren zu können. Unter Verwendung der Software Merlin (multipoint engine for rapid likelihood inference, Version 0.9.10) wurde nach signifikanten, nicht parametrischen LOD-Scores für die Kosegregation von Markerallelen mit der phänotypischen Ausprägung der sensorineuralen Taubheit beim Dalmatiner gesucht.

Ergebnisse

Die 24 ausgewählten menschlichen Kandidatengene für die kongenitale sensorineurale Taubheit beim Dalmatiner, wurden sowohl mittels der Fluoreszenz-*in-situ*-Hybridisierung als auch über das canine RH-Panel beim Hund kartiert. Für jedes Kandidatengen stimmten die

chromosomalen Lokalisationen dieser beiden voneinander unabhängigen Methoden überein (Tabelle 1). Die Kartierungsergebnisse entsprachen alle den Syntäniebeziehungen zwischen Mensch und Hund in der aktuellen vergleichenden Genomkarte.

Tabelle 1. Ausgewählte Kandidatengene mit entsprechender humaner und caniner Lokalisation.

Nr.	**Gen**	**humane**	**canine Lokalisation**	
		Lokalisation	**FISH**	**RH**
1	*CDH23*	10q21-q22	4q12-q13	4
2	*CLDN14*	21q22.3	31q15	31
3	*COCH*	14q11.2-q13	8q12-q13	8
4	*COL11A2*	6p21.3	12q11-q12	12
5	*DFNA5*	7p15	14q21.1	14
6	*DIAPH1*	5q31	2q23-q24.2	2
7	*EDN3*	20q13.2-q13.3	24q24-q25	24
8	*EDNRB*	13q22	22q21-q22	22
9	*EYA4*	6q23	1q13.3	1
10	*GJA1*	6q21-q23.2	1q24-q25	1
11	*GJB2*	13q11-q12	25q12-q21	25
12	*GJB6*	13q12	25q12-q21	25
13	*MITF*	3p14.1-p12.3	20q13	20
14	*MYH9*	22q12.3-q13.1	10q23.2	10
15	*MYO6*	6q13	12q21	12
16	*MYO7A*	11q13.5	21q13.3-q21	21
17	*MYO15A*	17p11.2	5q23-q24	5
18	*OTOF*	2p23.1	17q13	17
19	*PAX3*	2q35-q37	37q16-q17	37
20	*POU4F3*	5q31	2q21	2
21	*SLC26A4*	7q31	18q21	18
22	*SOX10*	22q13	10q21-q23	10
23	*TECTA*	11q22-q24	5q12-q13	5
24	*TMPRSS3*	21q22.3	31q15	31

Es wurden insgesamt 58 Mikrosatellitenmarker an Dalmatinerfamilien typisiert, wobei 50 Marker neu entwickelt waren und acht Marker der RH-Karte des Hundes entnommen wurden. Da sich einige Marker als homozygot, und daher als nicht informativ darstellten, konnten letztendlich 43 Marker (bestehend aus 36 neuentwickelten Markern und sieben Markern aus der RH-Karte) in der Analyse berücksichtigt werden. Das Markerset zeichnete sich durch eine durchschnittliche Allelanzahl von 3,5, einem PIC-Wert von 49%, einem H_E von 50,3% und einem H_O von 51% aus.

Für insgesamt fünf Marker, die zu zwei der oben genannten Kandidatengene assoziiert sind, konnte eine signifikante Kopplung, mit Irrtumswahrscheinlichkeiten von $p < 0,05$, zu der kongenitalen sensorineuralen Taubheit festgestellt werden. Für weitere vier Marker, die für zwei andere Kandidatengene genotypisiert wurden, lagen die Irrtumswahrscheinlichkeiten noch bei $p = 0,2$.

Anhand der berechneten Haplotypen konnte gezeigt werden, dass mit Ausnahme von einer einzigen Halbgeschwistergruppe, in allen untersuchten Dalmatinerfamilien entweder eines der identifizierten und signifikant gekoppelten Gene oder sogar beide Gene and der Entwicklung der Erkrankung beteiligt sein mussten.

Diskussion

Die Kombination des Kandidatengenansatzes mit der „affected-pedigree-member" Methode

In der vorliegenden Dissertation wurde ein Kandidatengenansatz mit der „affected-pedigree-member" Methode zur Durchführung einer Kopplungsanalyse kombiniert.

Aufgrund dieses neuen methodischen Ansatzes war es nicht notwendig, die genomische Struktur jedes einzelnen der 24 ausgewählten und kartierten Kandidatengene aufzuklären, sondern es müssen nun nur diejenigen Gene für weitere molekulargenetische Untersuchungen in Betracht gezogen werden, die eine signifikante Kopplung zu der kongenitalen Taubheit aufgewiesen haben. Durch die Entwicklung eines spezifischen kandidatengenassoziierten Markersets wurde die Möglichkeit einer Rekombination zwischen den Kandidatengenen und ihrer entsprechenden Marker weitestgehend minimiert. Daher war es möglich, die Kopplungsanalyse mit nur einem Bruchteil desjenigen Tiermaterials durchzuführen, welches für eine genomweite QTL-Studie erforderlich gewesen wäre.

Die Kartierung

Sowohl die Identität der isolierten kandidatengenenthaltenden Klone als auch die nachfolgenden Ergebnisse der Kartierungen wurden mit Hilfe zahlreicher unterschiedlicher Methoden überprüft. Die Kombination von der ECL-Hybridisierung mit dem Sequenzierung der BAC-Subklone und anschließendem Datenbankvergleich der Sequenzen, die Kartierung über zwei unabhängige Methoden, und letztendlich der Vergleich der Syntänieanaben zwischen Mensch und Hund, hat sich dabei als sehr wertvoll erwiesen.

Die chromosomale Lokalisierung von 24 caninen Genen ist ebenfalls ein wertvoller Beitrag für die Entwicklung einer hochauflösenden vergleichenden Genkarte von Mensch und Hund.

Das Markerset

Im Vergleich zu den Ergebnissen anderer Markerstudien beim Hund konnte der Informationsgehalt des entwickelten Markersets mit einem PIC-Wert von 49% als ausreichend informativ bewertet werden. Da für eines der Kandidatengene kein Marker mit einem ausreichend hohen PIC-Wert typisiert werden konnte, kann für dieses Gen eine mögliche Kopplung zur kongenitalen Taubheit nicht mit Sicherheit ausgeschlossen werden. Es ist hier empfehlenswert, über das Sequenzieren weiterer Subklone neue Mikrosatellitenmarker zu entwickeln und zu testen. Dies wäre eventuell auch für die sieben Kandidatengene denkbar, für welche nur jeweils ein informativer Marker in die Studie einbezogen werden konnte.

Mit dem entwickelten genassoziierten Markerset kann nun nicht nur weltweit bei allen Dalmatinerpopulationen, sondern auch bei allen anderen Hunderassen, die von kongenitaler sensorineuraler Taubheit betroffen sind, untersucht werden, ob eines oder eventuell mehrere der 24 ausgewählten Gene an der Krankheitsentwicklung ursächlich beteiligt sind. Die 36 neuentwickelten und physikalisch verankerten Marker dienen zudem der Verfeinerung der aktuellen RH-Karte des Hundes.

Die Kopplungsstudie

Die Ergebnisse der Kopplungsstudie ließen darauf schließen, dass mindestens zwei der untersuchten 24 Kandidatengene an der Entwicklung der kongenitalen sensorineuralen Taubheit in der untersuchten Dalmatinerpopulation beteiligt waren. Die beiden anderen Gene, deren Marker Irrtumswahrscheinlichkeiten von 0,2 erreichten, sind in Anbetracht des

insgesamt untersuchten Tiermaterials, ebenfalls noch als mögliche Kandidaten in Erwägung zu ziehen. Es ist daher anzunehmen, dass die kongenitale sensorineurale Taubheit beim Dalmatiner digen, wahrscheinlich sogar heterogen bedingt ist. Ebenso wiesen die Haplotypberechnungen darauf hin, dass sogar innerhalb verwandter Dalmatinerfamilien unterschiedliche Gene für die Ausbildung desselben Krankheitsphänotyps verantwortlich sein können.

Die signifikant gekoppelten Marker können bereits zur Durchführung eines indirekten Gentests verwendet werden. Über die Analyse der Haplotypen ist es mit diesem Gentest möglich, auf die hörenden Tiere zu selektieren, die das geringste Risiko haben, taubheitsverursachende Mutationen zu tragen und an ihre Nachkommen weiterzugeben.

Um die Rolle der beiden identifizierten Gene bezüglich der Taubheit letztendlich aufklären zu können, sollten diese Gene vollständig sequenziert und daraufhin auf kausale Mutationen für die phänotypische Ausprägung der Erkrankung untersucht werden. Die Ergebnisse einer solchen Mutationsanalyse könnten dann ebenfalls der Entwicklung eines direkten Gentestes für die kongenitale sensorineurale Taubheit beim Dalmatiner dienen.

Chapter 12

Appendix

Appendix 1. Characteritics for each marker including the number of alleles, h_O, h_E and the PIC.

marker	no. of alleles	h_O	h_E	PIC
CDH23_MS1	3	60.0	66.3	67.3
CDH23_MS2_F2	2	41.3	47.1	50.5
CLDN14_MS1	3	26.8	24.1	22.9
CLDN14_MS2	3	56.4	50.4	39.5
CLDN14_MS3	4	67.9	60.4	53.4
COCH_MS1	4	43.5	53.1	48.8
COCH_MS2	3	37.0	48.7	38.8
COL11A2_MS1	3	69.6	65.7	65.0
COL11A2_MS3	3	37.5	34.7	32.8
DFNA5_MS1	5	71.7	66.3	62.9
DFNA5_MS2	3	54.3	52	40.6
DIAPH1_MS1	5	67.4	61.3	56.2
DIAPH1_MS2	2	52.2	49.9	60.3
EDN3_MS1	6	73.9	70.8	67.5
EDNRB_MS1	2	31.1	32	30.9
EYA4_MS1	5	82.6	74.6	70.4
EYA4_MS2	2	56.5	49.6	58.1
GJA1_MS1	3	63.0	52.1	41.6
GJA1_MS2	2	55.6	50	62.5
GJB2_MS1	5	67.4	65.4	59.3
GJB2_MS2	2	39.1	40.5	40.3
GJB2+6_MS1	8	80.4	73.6	69.7
GJB6_MS2	7	80.4	77.7	74.3
MITF_MS2	2	19.6	21.1	20.3
MITF_MS3	3	19.6	23.5	22.5

Appendix 1. Continued

marker	no. of alleles	h_O	h_E	PIC
MYH9_MS2	3	17.8	16.4	15.4
MYH9_MS3	7	84.8	77.2	73.7
MYO6_MS2	2	53.3	45.8	48.0
MYO7A_MS1	2	26.7	26.3	25.2
MYO7A_MS2	2	30.4	40.5	40.3
MYO7A_MS3	2	28.3	39.6	39.1
MYO15A_MS1	4	47.7	62.4	59.8
MYO15A_MS2	2	52.2	49.9	60.3
OTOF_MS1	3	44.4	39.3	35.6
PAX3_MS1	3	32.6	30.1	28.4
PAX3_MS2	2	47.8	49.9	60.3
POU4F3_MS4	6	84.8	74.0	69.8
SLC26A4_MS2	3	47.8	62.4	58.2
SOX10_MS2	3	54.3	52.7	46.6
TECTA_MS1	2	4.4	4.3	4.3
TECTA_MS2	3	59.1	59.9	57.9
TMPRSS3_MS1	4	63.4	56	46.9
TMPRSS3_MS2	7	58.7	65.1	60.2

Appendix 2. Zmeans and LOD scores with their respective error probabilities for the gene-associated markers.

marker	All				Pairs			
	Zmean	**p_{Zmean}**	**LOD**	**p_{LOD}**	**Zmean**	**p_{Zmean}**	**LOD**	**p_{LOD}**
CDH23_MS1	0.01	0.5	0	0.5	-0.11	0.5	-0.01	0.6
CDH23_MS2_F2	0.01	0.5	0	0.5	-0.11	0.5	-0.01	0.6
CLDN14_MS1	1.00	0.2	0.63	0.04	1.06	0.14	0.49	0.07
CLDN14_MS2	1.02	0.2	0.64	0.04	1.08	0.14	0.49	0.07
CLDN14_MS3	1.02	0.2	0.64	0.04	1.08	0.14	0.49	0.07
COCH_MS1	-0.18	0.6	-0.01	0.6	-0.23	0.6	-0.03	0.6
COCH_MS2	-0.18	0.6	-0.01	0.6	-0.23	0.6	-0.03	0.6
COL11A2_MS1	1.41	0.08	0.85	0.02	1.66	0.05	0.67	0.04
COL11A2_MS3	1.31	0.1	0.78	0.03	1.51	0.07	0.62	0.05
DFNA5_MS1	0	0.5	0	0.5	0.06	0.5	0.01	0.4
DFNA5_MS2	-0.05	0.5	0	0.5	-0.1	0.5	-0.01	0.6
DIAPH1_MS1	-0.35	0.6	-0.01	0.6	-0.41	0.7	-0.05	0.7
DIAPH1_MS2	-0.35	0.6	-0.01	0.6	-0.41	0.7	-0.05	0.7
EDN3_MS1	-0.54	0.7	-0.02	0.6	-0.56	0.7	-0.07	0.7
EDNRB_MS1	0.13	0.4	0.08	0.3	0.1	0.5	0.03	0.4
EYA4_MS1	0.18	0.4	0.03	0.4	0.54	0.3	0.15	0.2
EYA4_MS2	0.19	0.4	0.03	0.4	0.54	0.3	0.15	0.2
GJA1_MS1	0.16	0.4	0.03	0.4	0.01	0.5	0	0.5
GJA1_MS2	0.08	0.5	0.01	0.4	-0.15	0.6	0.01	0.6
GJB2_MS1	0.55	0.3	0.14	0.2	0.59	0.3	0.11	0.2
GJB2_MS2	0.55	0.3	0.14	0.2	0.59	0.3	0.11	0.2
GJB2+6_MS1	0.55	0.3	0.14	0.2	0.59	0.3	0.11	0.2
GJB6_MS2	0.56	0.3	0.15	0.2	0.59	0.3	0.11	0.2
MITF_MS2	0.27	0.4	0.07	0.3	0.41	0.3	0.08	0.3
MITF_MS3	0.26	0.4	0.07	0.3	0.4	0.3	0.08	0.3

Appendix 2. Continued

marker	All				Pairs			
	Zmean	**p_{Zmean}**	**LOD**	**p_{LOD}**	**Zmean**	**p_{Zmean}**	**LOD**	**p_{LOD}**
MYH9_MS2	-1.05	0.9	0.03	0.7	-1.26	0.9	-0.16	0.8
MYH9_MS3	-1.06	0.9	0.03	0.7	-1.28	0.9	-0.17	0.8
MYO6_MS2	-0.26	0.6	-0.01	0.6	-0.33	6	-0.04	0.7
MYO7A_MS1	-0.46	0.7	-0.02	0.6	-0.72	0.8	-0.09	0.7
MYO7A_MS2	-0.46	0.7	-0.02	0.6	-0.72	0.8	-0.09	0.7
MYO7A_MS3	-0.46	0.7	-0.01	0.6	-0.72	0.8	-0.09	0.7
MYO15A_MS1	-0.12	0.5	0	0.6	-0.14	0.6	-0.01	0.6
MYO15A_MS2	-0.12	0.5	0	0.6	-0.13	0.6	-0.01	0.6
OTOF_MS1	-0.47	0.7	-0.02	0.6	-0.54	0.7	-0.07	0.7
PAX3_MS1	-0.37	0.6	-0.01	0.6	-0.35	0.6	-0.05	0.7
PAX3_MS2	-0.37	0.6	-0.01	0.6	-0.34	0.6	-0.04	0.7
POU4F3_MS4	0.19	0.4	0.02	0.4	0.25	0.4	0.02	0.4
SLC26A4_MS2	-0.36	0.6	-0.01	0.6	-0.44	0.7	-0.05	0.7
SOX10_MS2	-0.08	0.5	0	0.5	-0.42	0.7	-0.05	0.7
TECTA_MS1	0.18	0.4	0.06	0.3	0.4	0.3	0.14	0.2
TECTA_MS2	0.17	0.4	0.05	0.3	0.4	0.3	0.14	0.2
TMPRSS3_MS1	-0.15	0.6	0	0.6	-0.12	0.5	-0.01	0.6
TMPRSS3_MS2	-0.12	0.5	0	0.6	-0.09	0.5	0	0.5

Chapter 13

List of publications

List of publications

Journal articles

1. RAK, S. G., DRÖGEMÜLLER, C., KUIPER, H., LEEB, T., QUIGNON, P., ANDRÉ, C. & DISTL O. (2002): Cloning and chromosomal localization of MYO15A to chromosome 5 of the dog (Canis familiaris). Chromosome Res. 10, 407-10.

2. RAK, S. G., DRÖGEMÜLLER, C., KUIPER, H., LEEB, T., QUIGNON, P., ANDRÉ, C. & DISTL, O. (2002): Comparative mapping of the canine diaphanous homologue 1 (Drosophila) gene (DIAPH1) to CFA2q23-q24.2. Anim. Genet. 33, 389-90.

3. RAK, S. G., DRÖGEMÜLLER, C., LEEB, T., QUIGNON, P., ANDRÉ, C., SCOTT, A., BREEN, M. & DISTL O. (2003): Chromosomal assignment of 20 candidate genes for canine congenital sensorineural deafness by FISH and RH mapping. Cytogenet. Genome Res. 101, 130-35.

4. DRÖGEMÜLLER, C., RAK, S. G., KUIPER, H., LEEB, T., QUIGNON, P., GALIBERT, F. & DISTL, O. (2002): Assignment of the canine tectorin alpha gene (TECTA) to CFA5q12→q13 by FISH and confirmation by radiation hybrid mapping. Cytogenet. Genome Res. 97, 140A.

5. KUIPER, H., RAK, S. G., DRÖGEMÜLLER, C., LEEB, T., QUIGNON, P., GALIBERT, F. & DISTL, O. (2002): Assignment of the canine cadherin related 23 gene (CDH23) to chromosome 4q12→q13 by fluorescence in situ hybridization and radiation hybrid mapping. Cytogenet. Genome Res. 97, 140B.

Posters

1. RAK, S. G., DRÖGEMÜLLER, C., KUIPER, H., LEEB, T., QUIGNON, P., ANDRÉ, C. & DISTL, O. (2002): Mapping of candidate genes for non-syndromic sensorineural deafness in dogs. 28th Conference of the International Society of Animal Genetics in Göttingen, Germany, 11.-15.08.2002.

Oral presentations

1. RAK, S. G., DRÖGEMÜLLER, C., KUIPER, H., NOLTE, I., BULLERDIEK, J., LEEB, T., & DISTL, O. (2001): Kartierung von Kandidatengenen für die kongenitale Taubheit beim Dalmatiner. DGfZ/GfT conference in Weihenstephan, Germany, 12.–13.09.2001.

2. RAK, S. G., DRÖGEMÜLLER, C., KUIPER, H., LEEB, T., & DISTL, O. (2002): Molekulargenetische Analyse der kongenitalen sensorineuralen Taubheit beim Dalmatiner mittels Kandidatengenen. DGfZ/GfT conference in Halle (Saale), Germany, 18.–19.09.2002.

Chapter 14

Acknowledgements

Acknowledgements

First of all I wish to thank Prof. Dr. Dr. Ottmar Distl, the supervisor of my doctoral thesis, for offering me the opportunity to work on an exciting and challenging dissertation. His academic guidance, constructive criticism and support in the course of this work were invaluable.

Furthermore I am very thankful to my direct supervisor Dr. Cord Drögemüller and to Prof. Dr. Tosso Leeb for their great enthusiasm for my studies. They kept an eye on the progress of my work and were always available when I needed their advice.

My special thanks go to Prof. Matthew Breen and to Allyson Scott for their support regarding the FISH and for providing me beautiful images. Moreover, I want to thank Matthew for his ideas and encouragement during the development of our article.

I want to thank Dr. Heidi Kuiper for her assistance in the fluorescence *in situ* hybridisation.

I am also grateful to Catherine André, PhD, and Pascale Quignon, PhD, for the chromosomal assignment of genes by RH mapping.

I am very thankful to Heike Klippert-Hasberg and Stefan Neander for technical expertise and assistance.

I wish to express my appreciation to the Gesellschaft zur Förderung Kynologischer Forschung (GKF) e.V., Germany, for funding this work with a grant.

I am very thankful to all colleagues, Dalmatian breeders and owners for providing me blood samples and the results of the BAER tests.

I wish to thank Judith McAlister-Hermann, PhD, Markus James and Nicoletta Knorr for proof-reading this thesis.

I am appreciative to all friends and colleagues of the Institute of Animal Breeding and Genetics of the School of Veterinary Medicine Hannover for their support, friendship and humour. You all made me feel at home at work.

Last but not least I wish to thank Markus for his loving support.