



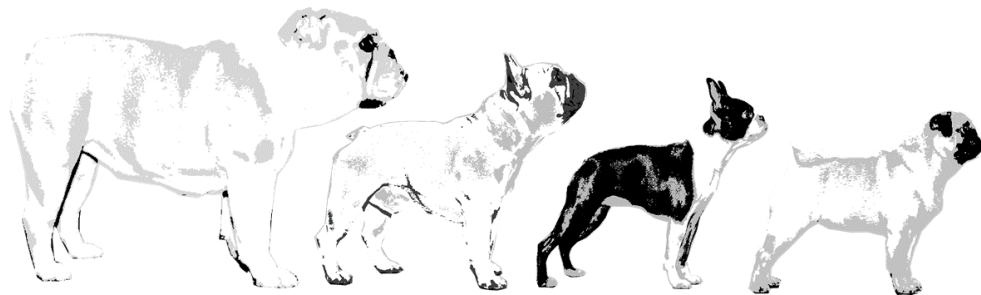
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Genetic variation in genes associated with canine brachycephaly

Genetisk variation i gener associerade med brakycefali hos hund

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Genetic variation in genes associated with brachycephaly

Genetisk variation i gener associerade med brakycefali

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Abstract

Domestication followed by controlled breeding has generated many dog breeds, displaying high morphological variation. One type of dogs with a distinct morphology is brachycephalic dogs, characterized by a shortened muzzle, a wide head and widely spaced eyes. The phenotype of brachycephalic dogs has been associated with several health issues, where one of the most obvious is the Brachycephalic Obstructive Airway syndrome (BOAS) which affects the breathing capacity and thermoregulation of the dog. Several studies have aimed to identify the molecular background of the shorter snouts in brachycephalic dogs. So far, mutations in genes such as Bone Morphogenic Protein 3 (*BMP3*), Fibroblast growth factor 4 (*FGF4*), SPARC-related modular calcium binding gene 2 (*SMOC2*) and DISHEVELLED 2 (*DVL2*) are thought to be associated with the altered skull shape causing canine brachycephaly. The aim of this master thesis was to investigate the genetic variation in the genes *SMOC2*, *BMP3*, and *DVL2* in the Swedish population of four brachycephalic breeds; Boston Terrier, English Bulldog, French Bulldog and Pug. 102 privately owned brachycephalic dogs were genotyped for the mutation in the *SMOC2* gene, additionally, 45 of these dogs were also genotyped for the mutations in the *BMP3* and *DVL2* gene.

The mutant variant of the *SMOC2* gene was fixated in all four breeds. The mutant variant of the *BMP3* gene had high allele frequency in all breeds. In Bull type breeds (Boston Terrier, English Bulldog and French Bulldog) the mutant version of the *DVL2* gene was fixated, whilst all Pugs were tested wild type for the *DVL2* mutation. Low genetic variation will make it more challenging to improve health through genetic selection within the breeds.

Keywords: Brachycephaly, dog, genetic variation, *SMOC2*, *BMP3*, *DVL*

Sammanfattning

Domesticering följt av kontrollerad avel har genererat många hundraser med stora variationer i utseende. En typ av hundar som uppvisar ett distinkt utseende är brakycefala (trubbnosiga) hundar, karakteriserat av en kort nos, brett huvud och med stort avstånd mellan ögonen. Fenotypen hos brakycefala hundar har kopplats till hälsoproblem där ett av de mest påtagliga problemen är Brachycephalic obstructive airway syndrome (BOAS), ett syndrom som påverkar hundens andning och förmåga att reglera sin kroppstemperatur. Fler-talet studier har utförts för att identifiera de molekylära bakomliggande orsakerna till den kortare nosen hos brakycefala hundar. Hittills har mutationer i gener så som Bone Morphogenic Protein 3 (*BMP3*), Fibroblast growth factor 4 (*FGF4*), SPARC-related modular calcium binding gene 2 (*SMOC2*) och DISHEVELLED 2 (*DVL2*) kunnat kopplas till den avvikande huvudformen hos brakycefala hundar. Syftet för detta examensarbete var att undersöka den genetiska variationen i generna *SMOC2*, *BMP3* och *DVL2* för den svenska populationen av bostonterrier, engelsk bulldogg, fransk bulldogg och mops. Totalt genotypades 102 privatägda brakycefala hundar för mutationen i *SMOC2* genen, dessutom genotypades 45 av dessa hundar för mutationerna i *BMP3* och *DVL2*. Denna studie pekar på att mutationen i *SMOC2* var fixerad i de fyra raserna. I de fyra raserna var allelfrekvensen för den muterade *BMP3* allelen hög. I de tre raserna av bull-typ (bostonterrier, engelsk bulldogg och fransk bulldogg) var mutationen i *DVL2* fixerad, medan alla mopsar var genotypade som vildtyp. Låg genetisk variation kommer göra det mer utmanande att förbättra hälsan genom genomisk selektion inom raserna.

Nyckelord: Brakycefali, trubbnos, hund, genetisk variation, *SMOC2*, *BMP3*, *DVL2*

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1 Introduction

1.1 The history of the dog

Man's best friend, the dog (*Canis lupus familiaris*), is one of the most popular pets today and is, in many cases, considered as a highly beloved family member. The origin of the dog, being the first animal to be domesticated, is thought to have begun around 11 000– 30 000 years ago (Thalmann *et al.* 2013; Freedman *et al.* 2014). Both the exact temporal and geographical origin of the dog is however controversial, and some studies suggests a single domestication event in East Asia while others suggest several domestication events in different geographic locations (Vila 1997; Frantz *et al.* 2016; Wang *et al.* 2016). While the answer to where and when the domestication occurred remains indefinite, genetic studies suggest that the dog descends from the gray wolf (*Canis lupus*) (Wayne 1993; Freedman & Wayne 2017).

The dog has evolved through a mutually beneficial relationship with humans, sharing food sources and living space. Modern dog breeds are the result of at least two genetic bottlenecks, where the population size has drastically increased. One bottleneck being the domestication event and the other through the man ruled intensive selection to create breeds (Lindblad-Toh *et al.* 2005). Dogs have been selectively bred to support human needs, such as hunting, herding, obedience, guarding, rescuing and for companionship. This selection has generated a large number of dog breeds, displaying large variety in behaviour, size, head shape, coat colour and coat texture (Lindblad-Toh *et al.* 2005; Schoenebeck & Ostrander 2013). Today, 346 dog breeds are recognised by the Federation Cynologique Internationale (FCI, 2019), spanning from the small Chihuahua to the big Great Dane, from the hairless Xoloitzcuintle to the long-coated Afghan hound, from the short-nosed Pug to the long-nosed Collie. No other domesticated species show such variations in phenotype and behaviour as the ones displayed among dog breeds (Stockard 1941).

The many dog breeds surely display a high morphological variation. For example, the skull shape can differ remarkably and might be a breed-defining feature. Skull shape can be divided into three categories, a short head which is technically called “brachycephalic” (e.g. Pug and French Bulldog), a long head which is technically called “dolichocephalic” (e.g. sighthounds and collies) and in between as an intermediate is the shape called “mesocephalic” or “mesaticephalic” (e.g. Beagle) (Evans & Miller 2013).

The morphological variation shown in the dog is a result of controlled breeding and the formation of breed standards. Typically, in the creation of a breed, a small number of dogs have been used. The small number of founders followed by a small number of males used for breeding, have reduced the effective population size in the dogs and created genetic drift, resulting in a reduced genetic diversity within breeds and a higher variation between breeds (Ostrander & Wayne 2005).

1.1.1 Genetic Diversity in dogs

The genetic diversity can be evaluated in several ways, for example by using genealogical data (i.e. pedigree data), where the average inbreeding coefficient (F) being one of the most widely accepted tools. At a population level, the inbreeding level must be evaluated with caution since “inbreeding” can have different meaning, depending on how its computed. When analysing pedigrees, the coefficient of inbreeding is often defined as the probability that two alleles at a given locus are identical by descent (IBD). Other useful tools for evaluating diversity when using pedigree data is the effective population size and ancestral constitutions (Leroy *et al.* 2009; Leroy 2011).

More recent ways to assess the genetic diversity within-breeds is the use of molecular data by using genetic markers (Leroy *et al.* 2009). Genetic markers like autosomal microsatellites, mitochondrial DNA, Y chromosome markers and Single nucleotide polymorphisms (SNPs) have been used to study genetic polymorphisms, degree of heterozygosity and phylogeny (Irion *et al.* 2003).

Irion *et al.* (2003) assessed genetic diversity by studying 100 microsatellites for 28 breeds recognised by the American kennel club (AKC). The breeds were selected from each of the seven recognised groups in AKC. An average of 41 dogs per breed was sampled and screened. By determining the allele frequencies and number of alleles at specific marker loci, the genetic polymorphism was determined. Total heterozygosity (i.e., the probability that two gametes randomly picked from a

population carries different alleles), the average heterozygosity among subpopulations and the average breed heterozygosity was calculated. The study showed that the total heterozygosity was high for all breeds, with an average of 0.618 and with only three breeds with an average breed heterozygosity below 0.5. It was also found that a decrease in population size and longer time since the breed recognition, decreased both average breed heterozygosity and number of alleles in Hardy-Weinberg equilibrium. After breed recognition, forces such as founder effects, bottlenecks like changes in population size and usage of popular sires will continue to decrease the genetic diversity in breeds (Irion *et al.* 2003).

Leroy *et al.* (2009) performed a study with the aim to assess the genetic diversity within dog breeds using both genealogical and molecular data. In total, 1514 dogs from 61 different breeds, representing all 10 groups (see supplementary information for details) recognised by Fédération Cynologique Internationale (FCI) was sampled and genotyped using 21 autosomal markers. The mean value for non-biased heterozygosity was calculated to 0.62 (Leroy *et al.* 2009), consistent with the result from the Irion study (Irion *et al.* 2003).

Pedersen *et al.* (2016) performed a study to assess the genetic diversity within the English Bulldog breed. The control group consisted of 102 bulldogs used for breeding and additional 37 bulldogs submitted for diagnostic tests was used as a case group. The study used several techniques to estimate the genetic diversity within the breed; sequencing the mitochondrial D-loop to decide maternal haplotypes, using 33 Short tandem repeat (STR) markers, in total seven Dog Leukocyte Antigen (DLA) class I and class II markers and lastly using six Y-specific markers for deciding paternal haplotypes. Additionally, SNP assay information from ten bulldogs and ten Standard poodles from other GWAS studies was used to obtain information of runs of homozygosity (ROH) in the breed. The observed heterozygosity for the English bulldog was calculated to 0.575 when including both cases and controls. By analysing the GWAS data, the alleles shared among all individuals, the runs of homozygosity were larger and involved more chromosomes when comparing the English bulldog to the Standard poodle. When analysing the maternal haplotypes, five haplotypes were identified. Three of the five haplotypes were identified in 90.9 % of the dogs in the control group, these three haplotypes were also found in other brachycephalic breeds, including mastiffs. For the paternal haplotypes, four haplotypes were identified. One of these, named Haplotype 1 was dominant and present in 93.1 % of the 44 males studied. Remaining haplotypes were observed at each 2.3 %. The dominant haplotype 1 were also found in breeds such as French Bulldog, Staffordshire Bull terrier, Miniature Bull terrier, Bull terrier, Beagle, Coton de Tulear and Mastiffs. The shared maternal and paternal haplotypes

between mentioned breeds might give an insight to the bulldogs' ancestry. For the STR markers, most loci had one or two alleles dominating in frequency, highest allele number was 11, whereas the lowest allele number was three. Out of 33 loci, 19 loci had a single allele with an allele frequency over 50 %, six loci with one allele with allele frequency of 70 % or higher and one locus fixed among almost all studied dogs. However, when analysing the internal relatedness (IR) implying how related the parents are and the effect on population fitness, the average IR for English bulldog was 0.007. As a comparison, a litter born from two sibling parents would have an IR of 0.25. When adjusting the IR using allele frequencies from village dogs, the IR increases to 0.34 implying a high degree of inbreeding. Village dogs have been shown to have genetic links to most modern breeds and since random breeding occurs in these types of populations, the population serves as a reservoir of ancestral genetic diversity inherited by descent. An increased adjusted IR was also observed in Standard poodles, but to a lower degree. The study indicates that the English bulldog has lost genetic diversity through small founder population and intensive phenotypic selection in breeding, which may make it hard to select for changes in the breed when it comes to healthier phenotypes (Pedersen *et al.* 2016).

Domestication followed by the formation of dog breeds with closed stud books, hard phenotypic selection, inbreeding and usage of popular sires have resulted in loss of genetic diversity. In some breeds, later bottlenecks where the population has decreased because of war, isolation or economic depression have also affected the genetic diversity (Ostrander & Wayne 2005). The high usage of popular males and a closed gene pool reduces the genetic diversity and increasing the incidence of inherited diseases within the breed. In many dog breeds, line breeding (i.e. mating between related individuals) have been carried out to maintain desired traits in the breed (Mellersh 2008). Loss of genetic diversity is not always correlated to increased disease incidence and poor health, but has been associated with unhealthy physiological and morphological traits in many dog breeds (Farrell *et al.* 2015). With low genetic variation within in a breed, it can be hard to eliminate deleterious traits in the population once recognized. A low genetic variation will also limit the ability to select for new traits or to, for example, improve unhealthy phenotypes (Pedersen *et al.* 2016).

1.2 Brachycephaly

The morphology of dogs with a short muzzle is highly deviant, both from other dog breeds but also from their ancestor, the wolf. The brachycephaly phenotype is characterised by a shortened muzzle, a wide head with widely spaced eyes and is often considered as “flat faced”, due to altered growth of basisphenoid and basioccipital bones in the skull (Packer *et al.* 2015). Brachycephalic breeds are for example Pug, Boxer, English bulldog, French Bulldog, Shih tzu and Japanese Chin. Some brachycephalic breeds, like the Bulldogs and the Boston terrier is thought to share a common ancestor. The early bulldog, often referred to as Bandogs, was used in bull-baiting and organized dog fights. These dogs were described as brave and brute dogs with huge jaws. Baiting with Bandogs were forbidden 1835 in England, and the number of Bandogs decreased drastically but the breed was rescued and refined, resulting in the modern bulldog (American Kennel Club).

The pug is, despite its resemblance with bulldogs, thought to be a very old breed descending from Asia (American kennel club).



Figur 1. A painting by Philip Reinagle from 1790, showing a Bulldog.

Many of the brachycephalic breeds are very popular world-wide as companion dogs. In Sweden, both the French bulldog and the Pug are popular breeds and were ranked number 10th and 16th respectively, in 2016 in number of registrations in the Swedish kennel club (SKK, 2016). Despite the popularity of these breeds, the brachycephalic phenotype is associated with several health issues.

A recent analysis of disease prevalence included more than 1.27 million dogs, thereof 184 748 brachycephalic dogs, the analysis was performed under a nine-year

period. The report identified some conditions more frequently occurring in brachycephalic dogs, compared to non-brachycephalic dogs. For example, the short nose combined with shallow orbits makes the eyes vulnerable, increasing the risk of eye injuries such as corneal ulcer, ocular traumas and conjunctivitis. The study showed that there is a three to four-time higher risk of cornea injuries in brachycephalic dogs, compared to non-brachycephalic dogs. A shortened skull and compact body may result in excessive skin and skin folds, increasing the risk for skin problems such as fungal skin diseases and pyoderma (Feng *et al.* 2017). However, one of the most obvious issues related to the brachycephaly conformation is the Brachycephalic Obstructive Airway Syndrome (BOAS), which predominately affects brachycephalic dogs. In a risk factor analysis performed by Njikam *et al.* (2009) it was found that brachycephalic dogs were 38 times more likely to have BOAS, compared to non-brachycephalic dogs (Njikam *et al.* 2009). The skull of a brachycephalic dog is significantly shorter, but the soft tissues of the head are not proportionally reduced as the skull. This is problematic, since the size reduced skull will contain too much soft tissue, which leaves little space for the passage of air (Harvey 1989). Consequently, BOAS is a respiratory syndrome where soft tissues blocks the dogs airways during respiration (Packer *et al.* 2015). BOAS is characterized by anatomical abnormalities such as elongated soft palate, hypoplastic trachea, narrowed nostrils and distortion of the pharyngeal soft tissues which causes resistance to airflow and restricted breathing (Fawcett *et al.* 2019). Breathing for brachycephalic dogs requires more laboured breathing to produce a higher negative pressure to cope with the resistance of the airflow (Koch *et al.* 2003).

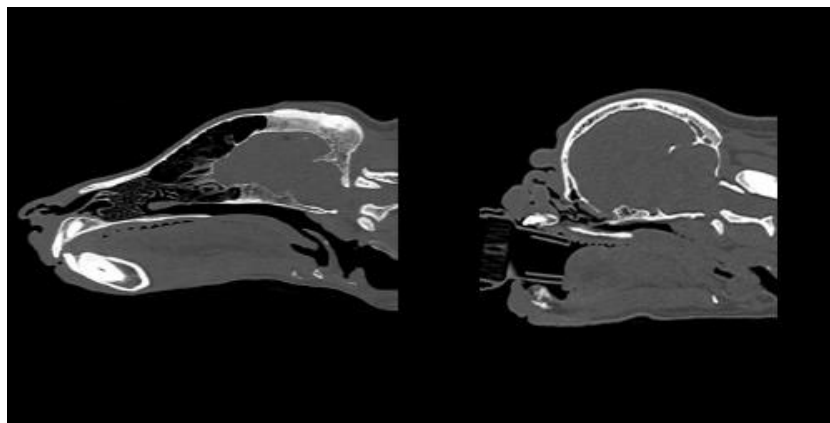


Figure 2. The figure shows Computed tomography scans of a German Shepherd (left) and a Pug (right). Picture: Cambridge University.

Clinical signs of BOAS includes noisy respiratory sounds such as stridor (wheezing) and inspiratory stertor (snoring), gagging, vomiting, regurgitation, syncope (fainting), dyspnoea (shortness of breath) and exercise intolerance (Riecks *et al.*

2007). The affected breathing might lead to increased sensibility to heat and impaired capability to thermoregulate (Davis *et al.* 2017), which might lead to a collapse when the dog is excited or hot (Koch *et al.* 2003).

A study performed by Packer *et al.* (2015) showed that the BOAS risk increases as the length of the muzzle shortens. In the 154 studied brachycephalic dogs, BOAS was only present in dogs with a muzzle length less than half of the cranial length (length from stop to the occipital protuberance). A thicker neck and obesity was also factors found to increase the BOAS risk (Packer *et al.* 2015).

Dogs affected of severe BOAS might, in some cases, need treatment such as weight loss, housing in cool environment and anti-inflammatory medicine. Some severe cases of BOAS might also require surgery (Riecks *et al.* 2007) in order to widen the nostrils or/and short the soft tissue blocking the airways. A recent study by Liu *et al.* (2017) investigated the outcomes and prognostic factors of surgical treatments in pugs, French and English Bulldogs. The dogs in the study had an improved respiratory function after surgery, although the respiratory function in 68 % of the dogs remained compromised. Age, the level of laryngeal collapse and body condition was found to be the main prognostic factors for BOAS surgery (Liu *et al.* 2017).

1.2.1 The Genetics behind Brachycephaly

Studies have revealed quantitative trait loci (QTL) on canine familiaris chromosomes (CFA) 1, 5, 18, 26, 32 and on the X chromosome, associated with the skull length in dogs (Boyko *et al.* 2010; Marchant *et al.* 2017). So far, mutations in genes such as Bone Morphogenic Protein 3 (*BMP3*), Fibroblast growth factor 4 (*FGF4*) and SPARC-related modular calcium binding gene (*SMOC2*) are thought to be associated with the altered skull shape causing canine brachycephaly (Boyko *et al.* 2010; Schoenebeck *et al.* 2012; Marchant *et al.* 2017).

SMOC2

Marchant *et al.*, 2017 found that the QTL on CFA1 was highly significant and associated with brachycephaly. A 187.7 kb critical interval in CFA1 was common among 30 out of 37 brachycephalic dogs participating in the study. Five variants were retained in this interval, one being a Long interspersed nuclear element (LINE-1) detected within the *SMOC2* gene. This LINE-1 insertion was predicted to cause a premature stop after exon 8 of the canonical 13-exon transcript, causing a fivefold reduction of the *SMOC2* mRNA expression. However, Marchant *et al.* (2017) claims that it remains unclear if the isoforms are translated but predicts that the protein

products would shear within the thyroglobulin-like domain, resulting in the protein missing the extracellular calcium-binding-domain. The study suggests that the insertion of the LINE-1 solely explains 36 % of the shortened face displayed in brachycephalic dogs in the study. By studying chick embryos, *SMOC2* expression was observed in the pharyngeal arches and shown to have temporal expression during the development of the later mandible and viscerocranium (Marchant *et al.* 2017).

Mansour *et al.* (2018) genotyped 109 brachycephalic dogs from eleven breeds; Boston Terrier, Boxer, Brussels Griffon, Bull Mastiff, Bulldog, Cavalier King Charles Spaniel, French Bulldog, Lhasa Apso, Pekingese, Pug and Shih Tzu for the *SMOC2* insertion. 80 of the 109 genotyped brachycephalic dogs was found to be homozygous, eight heterozygous (three Boston Terriers, three Bull Mastiffs, one Lhasa Apso and one Shih Tzu) and 21 dogs was found to be homozygous for the wild-type variant (one Boxer, six Bull Mastiffs, ten Cavalier King Charles Spaniel, two Lhasa Apso and two Pekingese).

The *SMOC2* gene is one of two homologues, where *SMOC1* and *SMOC2* are both members of Secreted protein acidic and rich in cysteines (SPARC) protein family, also known as *BM-40*. The members of the SPARC protein family, all contains an extracellular calcium-binding (EC) domain and a follistatin-like (FS) domain, (Termine *et al.* 1981; Maier *et al.* 2008). SPARC is a matricellular protein that is secreted into the extracellular matrix, but these proteins are not involved in primary structures in this location. Matricellular proteins like SPARC modulates cell function by interacting with hormones, proteases, cytokines and binding to growth factors and cell-surface receptors. SPARC proteins are expressed in high levels during embryonic development, in adult tissue both SPARC proteins are present in tissues undergoing repair or remodelling due to processes like wound healing or disease. (Bornstein 2009). *SMOC2* mRNA has been found to be expressed in many different tissues, such as spleen, skeletal muscle, ovary, testis, brain, thymus, lung, liver and in the heart (Vannahme *et al.* 2003). *SMOC2* has also been demonstrated to stimulate endothelial cell proliferation and migration, but also to be involved in formation of new capillaries (Rocnik *et al.* 2006).

Liu *et al.* (2008) investigated *SMOC2* expression in mouse embryos and found that *SMOC2* was distributed in the pharyngeal arches (i.e. the developing face), developing limbs and somites (Liu *et al.* 2008). Somites are epithelial cell clusters positioned bilaterally of the neural tube, which gives rise to cartilage, tendons, skeletal muscle, the vertebrae and ribs and the dermis of the back (Gilbert & Barresi 2016). Feng *et al.* (2009) examined gene expression during the development of the mouse facial prominences. Gene expression in the developing facial prominences

in mouse embryos were investigated between embryonic day 10.5 and 12.5 and it was found that several genes are expressed during the development, one gene being *SMOC2*, expressed in varying levels in samples from the maxillary, mandibular and frontonasal area (Feng *et al.* 2009).

In a case with dental defects in humans, Bloch-Zupan *et al.* (2011) found two cousins each homozygous for a mutation in the canonical-splice donor site in the first intron of the *SMOC2* gene. A zebra fish model was used to further investigate effects of mutations in *SMOC2* and the effect on dental development. Two types of morpholino knockdowns were created, one to target the initiation codon and one to target the boundary between exon 2 and intron 2 (Bloch-Zupan *et al.* 2011). Morpholino oligonucleotides (MO) is a tool to inhibit the translation of RNA transcripts. MOs is typically a oligomer of 25 morpholino bases that by complementary base pairing binds to the target RNA (Bill *et al.* 2009). When studying embryos carrying MO, the teeth were smaller with different shape and some dermal bones in the jaws and the fifth ceratobranchial bone were missing calcification, indicating that the skull is affected in morphants. Zebrafishes with the MOs also displayed a smaller head. Though, it should be noted that the development of teeth in zebra fish resembles the development in vertebrates, but the anatomy differs compared with for example humans. The knockdown of *smoc2* showed to effect expression of genes such as *dlx2b*, *bmp2a* and *pitx2*, all involved in tooth development (Bloch-Zupan *et al.* 2011).

Melvin *et al.* (2013) identified several genes that alter craniofacial conformation in zebrafish. Eight genes were fully analysed and grouped depending on what structure the alterations would affect; genes essential for formation of the ventral viscerocranium, genes essential for development of anterior neurocranium and genes critical for morphogenesis of all neural crest derived structures (including viscerocranium and neurocranium). *Smoc2* was analysed as one of the genes essential for the formation of viscerocranium. Two MOs were used to knockdown *smoc2*, one translation blocking MO to the 5' untranslated region (UTR) and one splice-blocking MO targeting a splice donor site for exon 11. Both MOs reduced the size of the head and eye abnormalities, 10 % in UTR MOs and 50-60 % of the splice site morphants were affected. The ceratohyals (hyoid horn) were flattened and inverted in morphants, compared to controls. The splice site donor MO altered the processing of *smoc2* RNA significantly, suggesting the observed affected craniofacial phenotype in morphants are due to loss of wild-type *smoc2* transcripts (Melvin *et al.* 2013).

The craniofacial defects investigated in these studies suggests that *Smoc* genes encode proteins that have a functional role in the development of the facial skeleton.

BMP3

By investigating the QTL at chromosome 32, a critical interval of 85 kb spanning the bone morphogenic protein 3 (*BMP3*) gene was found (Schoenebeck *et al.* 2012). After filtering, a Single nucleotide polymorphism (SNP) encoding for a missense mutation was found, nearly fixated among small brachycephalic dogs in the study. The mutation, called *BMP3^{F452L}*, is caused by a C to A transversion at position CFA 32:5231894, where C being the ancestral allele and A being the derived allele. The missense mutation causes a nonsynonymous substitution, changing the amino acid phenylalanine to leucine (Schoenebeck *et al.* 2012).

By using a zebra-fish model, *bmp3* mRNA expression was found as highly dynamic, first appearing during mid-somitogenesis (i.e. formation of somites) and later expressed throughout the head, brain, ventricles and posterior somites. 48 hours after fertilization, the *bmp3* expression is found in pectoral fins, the pharyngeal arch region, in the jaw structures and in the heart. The early expression of *bmp3* in the cranial structure suggested a role for Bmp3 in craniofacial development. By knocking down endogenous Bmp3 activity using translation-blocking antisense MO, it was found that MO treated zebrafish embryos demonstrated severe deficiency in the jaw development. Multiple cartilage elements that forms the viscerocranium and neurocranium were lost or underdeveloped in knocked down embryos. The results of the study indicates that Bmp3 is required for craniofacial development in zebra fishes and that the identified *BMP3^{F452L}* gene variant influences skull shape in brachycephalic dogs. (Schoenebeck *et al.* 2012).

Bone morphogenic proteins (BMPs), being multi-functional growth factors, belongs to the transforming growth factor β superfamily (TGF β). The TGF β pathway plays a central role in signalling networks controlling bone development, tissue repair, cell growth, cell differentiation and cell proliferation in metazoans. The TGF β family can be divided into several subfamilies, including protein families such as TGF- β , BMPs, activin and growth differentiation factors (GDF). TGF β initiate signalling through specific interactions with receptors on the surface of target cells (Massagué *et al.* 2000).

BMPs are involved in embryonic development and in multiple different cellular function in adult animals. BMP signalling is involved in heart, neural and cartilage development. It also plays an important role in the postnatal bone formation (reviewed in Chen *et al.*, 2004). BMP3, also known as osteogenin, works as an antagonist for other BMPs. BMP3 antagonises the ability of BMP2 to induce differentiation and cell commitment of osteogenic cells. Bmp3 is suggested to negatively affect bone density, where *Bmp3* null mice (*Bmp3^{-/-}*) showed to have twice as much

trabecular bone (spongy bone), compared to non-mutated littermates. Though, no differences in the size of the femur were observed and there were no difference in bone density between wild-type and heterozygous littermates (Daluiski *et al.* 2001).

By studying *Xenopus* embryos, Gamer *et al.* (2005) found that BMP3 inhibits activin and BMP-4 signalling. The activin type II receptor (ActRII) is a common receptor that binds to multiple ligands, such as activin and several BMPs. Binding between ActRII, activin or BMPs is required for patterning during the development of germ layers in the embryo. The binding between activins and BMPs and the receptor phosphorylates receptor regulated Smad proteins (R-Smad), R-Smad together with other transcriptional regulatory proteins regulates transcription of target genes. It was showed that if BMP-3 binds to its receptor (ActRII), BMP-3 works as an antagonist against activin and BMP-4. BMP-3 reduces the phosphorylation of R-Smad, and thereby inhibits the mesoderm-inducing activities of activin and BMP-4. By injecting BMP-3 in *Xenopus* embryos, overexpression of BMP-3 presents a phenotype with deviant tail formation, a shortened and curved body, reduced eyes and head structures and enlarged cement glands. However, the overexpression of BMP-3 could be rescued by co-injections of a truncated type II activin receptor (xActRIIB), which rescued the body axis and head structure in a dose dependent manner (Gamer *et al.* 2005).

FGF4

In the study by Marchant *et al.* (2017), a retrogene for the Fibroblast growth factor 4 (FGF4) located on chromosome CFA 18 was associated with the size of the neurocranium centroid size in brachycephalic dogs (Marchant *et al.* 2017). Earlier studies on FGF4 by Parker *et al.* (2009) identified the FGF4 retrogene on chromosome 18 that causes short legs (canine chondrodysplasia), present in dog breeds such as Dachshunds and Corgis (Parker *et al.* 2009).

The fibroblast growth factor family, composed of several secreted proteins, functions in the earliest stages of the embryonic development, organogenesis and in adult tissue where they act as homeostatic factors important for maintenance of the tissue, tissue repair and regeneration and metabolism. FGFs signals to receptor tyrosine kinases and intracellular non-signalling proteins (i.e. intracellular FGFs) that serves as cofactors. The FGF family is conserved and can be found in both invertebrates and vertebrates and is expressed in almost all tissues (reviewed in Ornitz & Itoh, 2015).

By investigating RNA expression in mice, Niswander and Martin (1992) found that *Fgf-4* expression is important during early embryonic development. *Fgf-4*

mRNA could be detected at late blastocyst stage in cells giving rise to embryonic lineages. Later, in early gastrulation, *Fgf-4* was expressed in the primitive streak where definitive endoderm and mesoderm are formed. After the establishment of the three germ layers, *Fgf-4* mRNA was detected in branchial arch units, somite myotome and in the apical ectodermal ridge where the limb buds develop. Expression was also found in tooth bud, suggesting that *Fgf-4* has multiple roles during the development of the embryo.

Boulet et al., (2004) studied *Fgf8* and *Fgf4* in mice embryos and found that mice lacking *Fgf4* in apical ectodermal ridge had normal limbs. In *Fgf8* mutants, the limb development was severely affected, with some skeletal elements of the limb missing. When creating mutants lacking both *Fgf4* and *Fgf8* in the forelimb apical ectoderm ridge, the forelimb does not develop, due to that the limb mesenchyme fails to survive in absent of both FGFs. When inactivating *Fgf4* and *Fgf8* in both forelimb and hindlimb, all limbs fails to develop. It was suggested that *Fgf8* is necessary for maintenance or initiation of Sonic hedgehog (*Shh*) and that *Fgf4* partially can compensate for the loss of *Fgf8* in development of the distal limb (Boulet *et al.* 2004).

FGFs is also thought to be involved in the development of the hair follicle. Salmon Hillbertz et al. (2007) found that ridged dog breeds, such as Rhodesian Ridgeback and Thai Ridgeback, are either heterozygous or homozygous for a large duplication. The duplication that is thought to be the causative mutation for the ridge, included genes such as FGF3, FGF4, FGF19, ORAOV1 and the 3' end of CCND1, encoding for cyclin D1. The ridge also predisposes dermoid sinus, a congenital developmental disorder (Salmon Hillbertz *et al.* 2007), caused by an incomplete separation of the neural tube and the skin .

DVL2

A recent study by Mansour et al. (2018) identified a frameshift mutation in the *Wnt* pathway gene Dishevelled Segment Polarity Protein 2 (*DVL2*) located on canine chromosome CFA 5, associated with the screw tail that is characteristic for English bulldogs, French bulldogs and Boston terriers. The frameshift mutation, caused by a deletion of a C (g.32195043_32195044del), preserves the majority of the *DVL2* protein, though the last 48 amino acids at the C terminus of the protein is replaced by novel sequence of 26 amino acid residues. The results indicated that the *Wnt*-dependent phosphorylation of the mutant variant *DVL2* protein was reduced, compared to the wild type variant. Out of 177 dogs from screw tailed breeds, 171 dogs were found homozygous for the mutation in *DVL*. The exception was six Boston Terriers, where four individuals were heterozygous, and two individuals were homozygous wild type. The pugs tested was found to be of wild types, as expected,

since the tail of a pug is of full length without malformations of the caudal vertebrae (Mansour *et al.* 2018). Mutations in Dishevelled genes in humans is characterised by craniofacial, limb and vertebral malformations, causing a syndrome named Robinow syndrome. Affected individuals often displays distinctive facial features such as widely spaced eyes, a wide forehead, short nose and a broad mouth (Genetics Home Reference 2019). Due to the resemblance between the phenotype of humans affected by the Robinow syndrome and the phenotype displayed in brachycephalic dogs, it is possible that the DVL2 mutation in screw tail dogs also have an effect on the brachycephalic phenotype (Mansour *et al.* 2018).

The first dishevelled mutation was identified in *Drosophila*, where a viable allele was associated with a phenotype with disoriented hairs on the body and wings. It was later shown that Dishevelled being a signalling molecule, works as a key component in the Wnt signalling pathway (Boutros & Mlodzik 1999).

Wnt proteins are one of the major families of developmentally important signalling molecules, involved in processes such as embryonic induction, generation of cell polarity and cell fate specification during embryonic development and tissue homeostasis. It is also suggested that Wnt signalling is involved in cancer genesis in humans (Cadigan & Nusse 1997). One of the best understood Wnt gene is the wingless (*wg*) gene, essential for segmentation and patterning events in the fruit fly *Drosophila melanogaster*. Wg is required in several events during embryo development, such as in patterning of the segmented ectoderm in the trunk, the development of the head, the central nervous system and the legs. Wg is also required for growth of the wing blade in the development of the wing disc, hence the name wingless. In the adult fly, Wg is required for patterning of bristles and body hair, in antennae, eyes and genitalia (reviewed in Klingensmith & Nusse, 1994).

Wnt genes have been identified in invertebrates and vertebrates, with ortholog relationship between species such as *Wnts* in *Drosophila* and in humans. Between vertebrates, the *Wnt* genes are highly conserved, sharing sequence identity and gene structure. It has been showed that signaling by members of the *Wnt* family have diverse functions during embryogenesis, and has been suggested that *Wnt* genes play roles in the development of different structures of the brain, development of dorsal-neural tube derivatives, development of kidneys, hair growth, limb polarity and the development of the placenta. Mutations in *Wnt* genes has also been associated with shortened anterior-posterior axis and female infertility due to defects during development of female reproductive organs (rewieved in Miller, 2001). In mouse, mutations in *Wnt* genes have been associated with defects in the tail, the tailbud and in caudal somites (Cadigan & Nusse 1997).

The Wnt pathway is activated when the Wnt ligand binds to the two membrane receptors, Frizzled (Fz) and low-density lipoprotein receptor related protein 6 (LRP6) or LRP5. The complex of ligand and receptors, together with the recruitment of Dishevelled results in phosphorylation of LRP6/LRP5 and the recruitment of the axin complex to the receptors. Next, the axin complex is inhibited, which allows β -catenin located in the cytoplasm to enter the nucleus and form complexes with DNA-bound T cell factor/lymphoid enhancer factor (TCF/LEF), where β -catenin serves as a co-activator which activates Wnt target gene expression. In absence of Wnt, β -catenin in the cytoplasm is constantly degraded by the axin complex and the Wnt target genes are repressed (MacDonald *et al.* 2009). Proteins encoded by the dishevelled genes works as important transducers in Wnt pathways, that conducts the Wnt signal to downstream cellular machinery (Na *et al.* 2007).

The Dishevelled proteins have three known structural domains, DIX (Dishevelled and Axin) domain at the N-terminus, PDZ (Post-synaptic density protein-95) and DEP (Dishevelled, Egl-10 and Pleckstrin). By investigating DVL2, Gammons *et al.* (2016) found that the DEP domain, binding to Frizzle receptors is essential for the Wnt-dependent signaling to β -catenin. The PDZ domain was dispensable for the β -catenin response (Gammons *et al.* 2016).

Tadjuidje *et al.* (2011) showed that both Dvl2 and Dvl3 plays a part in early embryo development in the *Xenopus*. It was found that both Dvl2 and Dvl3 are maternally encoded for. Before the zygotic gene activation (ZGA), where the embryo starts to produce its own transcripts and proteins, development is dependent of stored maternal factors in the oocyte. Genes encoding for such factors are referred to as maternal effect genes, are important for the oogenesis, enables ZGA and the progression of the early embryo development (Kim & Lee 2014). By studying *Xenopus* oocytes, it was found that *Dvl2* and *Dvl3* mRNA are expressed early in the development and decreases during the developmental process. *Dvl2* was later found to increase after the ZGA when the zygotic transcription starts, but *Dvl3* remained low. *Dvl1* was not detected until later in the development. By using different anti-sense oligos to block translation of Dvl2 and Dvl3, it was found that maternal depletion of Dvl2 or Dvl3 results in similar phenotypes. Depletion of Dvl resulted in shortened body axis, blastopores not closing and open neural folds. The heads were also found to be smaller in Dvl depleted embryos. Since maternal depletion of either Dvl2 or Dvl3 affects early development, it was suggested that Dvl2 and Dvl3 are not redundant to each other and that the proteins might interact with each other. Dvl2 and Dvl3 was found to be required for activation in the c-jun N-terminal kinase

(JNK) pathway which is important for the convergence extension (Tadjuidje *et al.* 2011), where cell movements results in elongation of the body axis.

Xing *et al.* (2018) studied the effects of mutations in zygotic Dvl, maternal Dvl and the effects when combining mutations in both zygotic and maternal Dvl in zebrafishes. By generating mutated lines for the five known *dvl* genes in zebrafish, it was found that only *dvl2* and *dvl3* were maternally expressed. Maternal and zygotic mutants for four out of five of the *dvl* genes developed normally and survived to adult zebrafish. *Dvl2* mutants were however found to have defects. Zygotic *dvl2* (*Zdvl2*) mutants displayed a slightly reduced anterior-posterior axis, compared to wild type embryos. Half of the *Zdvl2* mutants survived to adult, one third of the surviving females could not spawn and all male mutants were not able to produce offspring due to absent courtship behaviour. Embryos with mutations in both maternal and zygotic *dvl2* (*MZdvl2*) developed craniofacial defects, fused eyes and the pharyngeal cartilages protruded outwards. *MZdvl3* embryos displayed just a weak axis extension defect. When generating double heterozygote, (*dvl2*^{+/-}; *dvl3*^{+/-}), these embryos showed a slight reduction of the axis extension. However, when mating two double heterozygous fishes, embryos displayed a shortened anterior-posterior axis, a compressed head and reduced swim bladder. Absence of zygotic Dvl2 and Dvl3 resulted in moderate convergence extension defects, while absence of both zygotic and maternal Dvl3 and Dvl3 generated severe convergence extension defects. Progressive reduction of Dvl dosage was found to gradually induce anterior-posterior patterning defects, ranging from posterior deficiency to complete lack of the trunk and tail. In conclusion, Xing *et al.* (2018) found that Dvl3, and in particular Dvl2, is important in the convergence extension movement and in anterior-posterior patterning during embryonic development (Xing *et al.* 2018).

1.3 Aim of study

The aim of this thesis was to investigate the genetic variation in three of the genes associated with canine brachycephaly by looking into the Swedish population of four brachycephalic dog breeds; English bulldog, French bulldog, Pug and Boston terrier.

2 Material and Method

2.1 Recruitment of dogs

In total, data from 102 privately owned brachycephalic dogs (44 males and 58 females) were used in this thesis. The dogs were phenotypically described at six gatherings, held between 17th of September 2018 and 20th of January 2019. Three of the gatherings were organised by the Swedish kennel club (SKK), one gathering was organised by SKK and the English Bulldog club and two were organised by SKK and the French Bulldog club. The gatherings held by SKK and SKK/English Bulldog club were held at SKKs facility, while gatherings organised by the French Bulldog club were held in dog training facilities at two different locations.

Information from 18 English Bulldogs (9 males and 9 females), 48 French Bulldogs (23 males, 24 females and one with unknown sex), 21 Pugs (7 males and 14 females) and 15 Boston terriers (5 males and 10 females) were used for this thesis. The dogs in the inventory was between 9 months and 13 years old. In addition, DNA from one Labrador Retriever, one Golden Retriever, two Pugs and two Chinese Crested Dogs were used.

2.1.1 Swedish inventory of Brachycephalic dogs

The data used in this project was collected as part of a bigger Nordic research study with the aims to investigate the phenotypic and genotypic variation in the four breeds; English Bulldog, French Bulldog, Pug and Boston terrier, and if it's possible to by selection achieve changes in anatomy to reduce the predisposition of BOAS. The ambitions in the Swedish project was to recruit 50 – 100 dogs of each breed, approximately 25 dogs of each breed should later be selected and genotyped. The inventory is a part of the SKK's ambition to reduce extreme conformations in

Swedish dogs. At the gatherings, the procedure consisted of four stations. Station one, where the dog owner filled in information of the dog, such as birth date, sex, registration number, medical information and gave consent to participation in the study. At the second station, a veterinary inspection was performed to record measurements of the dog and parameters such as respiration and circulation. Some of the measurements taken is illustrated in figure 3 below.

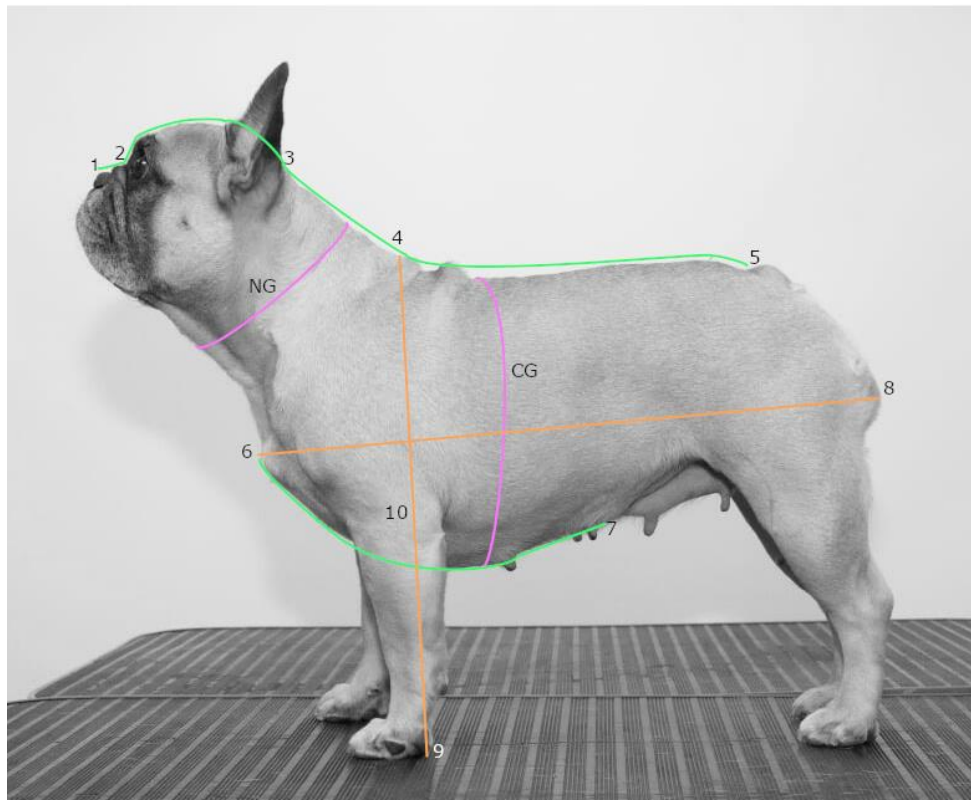


Figure 3. Illustration of some of the measurements taken during the inventories (Photo and illustration, Elin Johansson).

The skull length was measured from the stop (2) to the external occipital protuberance at the back of the skull (3). The snout length was measured from the stop (2) to the nose tip (1). Other measurements taken were the neck girth (NG), neck length (3 to 4), chest girth (CG), back length (4 to 5), body length (6 to 8), height at withers (4 to 9), elbow height (10 to 9) and chest length measured between the top of the breast bone (6) and the end of the sternum (7). By dividing the snout length with the skull length, the craniofacial ration was obtained. The degree of nostril stenosis was also graded according to a four graded scale from open nostrils to severe stenosis, described by (Liu *et al.* 2017). The dogs were also weighed, and the body condition

score was assessed, were a scale from one (undernourished) to nine (obese) was used. The dog's skin and presence of excessive skin folds were also examined.

At a third station, DNA was collected, and the dog was photographed from different angles (profile, front, back and from above). At the last station, additional conformation traits were recorded, such as height at shoulders, height at elbow, back length, body length, neck girth, chest girth and chest length. The placement and appearance of eyes, nose and ears and front and rear angles, appearance of paws, top line, wrinkles on body and nose folds and the length of tail and its appearance were also assessed. The dog's movement was scored when letting the dog trot back and forth on the floor.

Each dog was given a specific identification number at the gathering, partly to easy have access to an overview of the number of dogs in the inventory and partly to handle the information more objectively by just using the inventory number and/or breed information.

A second master thesis was done within the same Swedish project, with the aim to investigate the phenotypic variance correlated to BOAS in the Swedish population of four brachycephalic breeds; English Bulldog, French Bulldog, Pug and Boston Terrier and discuss their welfare implications (Bertilsson, 2019).

2.2 DNA extraction and quantification

Buccal cells were collected from the participating dog's cheeks by two or three bristle cytology brushes. The cytology brushes were left to air dry for a couple of minutes and later placed in a marked paper envelope. The envelopes with the cytology brushes was then stored in room temperature until DNA extraction. The envelopes were stored between one day and four months, depending on when the sample was collected and when the extraction was performed.

Genomic DNA was isolated using the QIA Symphony® SP automated system with the QIA Symphony DSP DNA Midi Kit (QiaGen, ID 937236, Hilden, Germany) (protocol in appendix I).

To determine the nucleic acid concentration in the samples, spectrophotometry was used by loading 2 µl of the samples on a NanoDrop™ 8000 Spectrophotometer (Thermo Fisher Scientific), this was performed on eight of the samples. Due to the large variation in nucleic acid concentration and the uncertainty of how much of the

nucleic acid is derived from dog's own buccal cells and how much is from bacteria or feed residues in the mouth, it was decided not to use the spectrophotometer on the other samples.

To ensure that the samples contained sufficient amount of canine genomic DNA, a polymerase chain reaction (PCR) of the canine housekeeping gene Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was set up, using the HotStarTaq® kit (QiaGen ID: 203601, Hilden, Germany) (protocol 1, Appendix A). In total, the PCR was set up for nine samples, including eight brachycephalic dogs and one Labrador Retriever. A gel electrophoresis was performed to segregate the PCR products for validation.

A dilution series was performed to investigate which concentrations that could work for a PCR. Three samples were picked out, based on the nucleic acid concentration measured with NanoDrop™, one sample with high, intermediate and low concentration was chosen. The samples were diluted to concentrations 1:10 and 1:100 and a second PCR for the *GAPDH* gene was set up (Appendix A, protocol 1). This procedure was mainly performed to assess the least possible amount of genomic DNA to use from each sample due to the expected limited amount of genomic DNA template obtained from the cheek swab samples available in this study.

In total, DNA from five additional dogs were used in the study as positive controls. DNA from one Labrador Retriever, one Golden Retriever, two Pugs and two Chinese Crested Dogs. For the Retrievers and the additional Pug, the DNA extraction was made from whole blood. For the two Chinese Crested Dogs, cytology brushes were used as described for the brachycephalic dogs above.

2.3 PCR amplification

2.3.1 Primer design

In order to detect the LINE-1 insertion in the *SMOC2* gene, PCR primers for genotyping described in Marchant et al. (2017) was used. The primers were checked, using the Primer3 software. Primers were obtained from TAG Copenhagen A/S (Denmark).

In total, three primers were used (table 1). All three primers were used in the same reaction. The melting temperatures of the primers were between 55.3°C and 60°C, with a GC content between 47.8% and 70 % (Appendix A, protocol 2).

Table 1. *Primers used for amplification of SMOC2.*

Primer	Primer sequence	Size
SMOC2 F	GGC AGG GGA TGG GGA AGC CT	531 bp
SMOC2 R (wild type)	ACT GTG TGC TTT GCC CAA ACT CA	
SMOC2 F	GGC AGG GGA TGG GGA AGC CT	698 bp
SMOC2 R (mutated)	TGC CCA TAA AGT TCA GGG TCC ACT	

Heterozygous will show as two visible bands on an agarose gel.

2.3.2 PCR conditions

For 102 dogs, the DNA sequences containing the SMOC2 gene was amplified using the kit HotStarTaq®. The total reaction volume of the PCR was 10 µL, composed of; 1x PCR Buffer (15 mM MgCl₂, ph 8.7) + dNTP [0.25 mM] + primers [0.3 µM/primer] + 0.125 µL Taq Polymerase [0.025 U/µL] + 1 µL DNA and addition of RNase-free water until the completion of the 10 µL reaction volume. One negative control per master mix was included, where the DNA was substituted with 1 µL of water (Appendix A, protocol 3)

PCR reactions were performed in a thermal cycler (ProFlex PCR system, Applied Biosystems by Life Technologies), using a touchdown PCR program, according to following protocol. Initiation step: 95 °C for 2 minutes. 3-step cycle (20 cycles) Denaturation step: 98 °C for 10 seconds. Annealing 72 - 52 °C (reduced with 1 °C/cycle) for 30 seconds. Extension: 72 °C for 45 seconds. Followed by another 3-step cycle; 98 °C for 10 seconds, 55 °C for 30 seconds, 72 °C for 45 seconds. Finishing synthesis step: 72 °C for 7 minutes. The lid heat was put on 98 °C. Thereafter, infinite hold in 4 °C until the products were quantified on an 2 % agarose gel, using a 100 bp precision size marker. The gel electrophoresis was run in 110V for approximately two to three hours, depending on the size of the gel.

The PCR protocol was kindly provided through personal communication with Marchant, T (March 2019).

2.4 Sanger Sequencing

Since no dog in this study had a known genotype for the *BMP3* and *DVL2*, Sanger Sequencing with capillary electrophoresis was performed to obtain the genotype for

three dogs, one Golden Retriever, one English Bulldog and one Pug, and use these dogs as positive controls in later TaqMan assays. Two sets of M13 tailed primers per gene, flanking the SNP were used (table 2).

Table 2. Primers used for sequencing *BMP3* and *DVL2* genes.

Primer	Primer sequence	Source
BMP3 1 F	GGTGATGATACAGGAGATTGTGCCAAA	Schoenenbeck et al., 2012
BMP3 1 R	CTGCCAGGTTATCTGCAAGCACAAG	Schoenenbeck et al., 2012
BMP3 2 F	TCATGCCACCATCCAGAGTA	Primer 3
BMP3 2 R	GCCAGGTTATCTGCAAGCAC	Primer 3
DVL2 1 F	CGGCTAGCTGTCTAGTTCTGG	Mansour et al., 2018
DVL2 1 R	CAGTGAGTCTGAGCCCTCCA	Mansour et al., 2018
DVL2 2 F	CCTGGGCTCCATCCCTAT	Primer 3
DVL2 2 R	GCGCCCTACATAACATCCAC	Primer 3

PCR reactions were performed according to the BigDye® Direct Sequencing Kit Protocol (Applied Biosystems®, Inc) (see Appendix A, protocol 3). PCR reactions were performed in a ProFlex™ PCR system (Applied Biosystems®), later the PCR-amplicons were purified using the BigDye Directs Cycle Sequencing Kit, following manufacturer's instructions. Templates were prepared to be sequenced both in forward and reverse orientation. PCR sequence products were analysed on the ABI 3500XL Genetic Analyzer (Applied Biosystems®).

2.5 TaqMan assays

For genotyping of the SNPs in the *BMP3* and *DVL2* gene, TaqMan® SNP genotyping assays were used. In total, 45 brachycephalic dogs and one non-brachycephalic dog (25 females, 20 males, 1 unknown) were genotyped for polymorphism in each of the two genes, *BMP3* and *DVL2*. The genotyping assays were designed according to custom manufacturer specifications.

The reaction was carried out in a 96 well plate. The reaction volume was 15 µL per well and was consisting of 7.5 µL TaqMan® Universal PCR Master Mix [2X], 0.38 µL TaqMan® Genotyping assay mix (primers + probes) [40X], 5.62 µL DNase-free water and 1.5 µL DNA template. The software StepOne™ Software v2.3 and the instrument StepOnePlus™ (Applied Biosystems®) Real-time PCR system generated the results. Cycle conditions according to following protocol: Pre PCR stage in 50 °C for 2 minutes, polymerase activation in 95 °C for 10 minutes,

followed by a touch-down series starting with denaturation in 95 °C for 15 seconds followed by cycling stage of 50 cycles with annealing/extension in 60 °C/55°C for *BMP3* and *DVL2* respectively, followed by 95°C for 15 seconds and an annealing/extension step in 55°C for 1 minute. Finally, a hold stage of 60 °C for 30 seconds.

The TaqMan assay relies on two sequence specific primers (forward and reverse) for PCR amplification. The assay also contains two allele specific TaqMan probes, each probe can detect one of the two alleles. The probes carry a dye label at the 5' end, a so called “reporter”. Commonly, VIC® reporter is used for allele one and FAM™ reporter for allele two. At the 3' end, the probe carries a non-fluorescent quencher bound to a minor groove binder (MGB). Each probe anneals specifically to the complementary sequence, depending on which allele is present. At denaturation, the primer pair and the corresponding probe anneals to the sequence. At polymerisation, probes hybridized to the target gets cleaved. The cleavage separates the reporter dye from the quencher and increases the fluorescence of the reporter, which can be detected by the device. The fluorescence generated during PCR amplification indicates which of the alleles that are present in the sample (ThermoFisher Scientific).

For genotyping of *BMP3*, VIC® reporter was used to detect the wild-type allele C (allele 1) and FAM™ reporter was used to detect the derived A allele (allele 2). For genotyping of *DVL2*, VIC® reporter was used to detect C allele (allele 1) for wild-type and FAM™ reporter was used for the derived A allele (allele 2).

The dogs with known genotyped was used as positive controls, other marked as unknown. After sequencing, the results were analysed using StepOne™ software and the ThermoFisher Cloud.

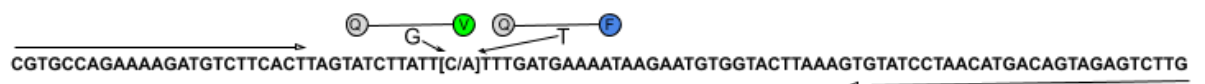


Figure 4. Schematic figure of the TaqMan assay for BMP3. Primers are marked out as arrows.

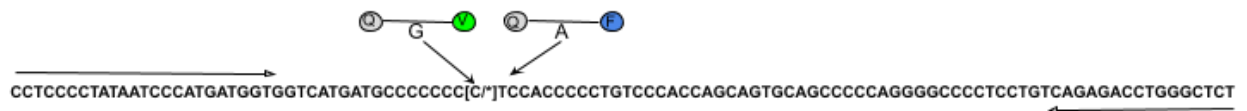


Figure 5. Schematic figure of the TaqMan assay for DVL2. Primers are marked out as arrows.

3 Results

3.1 GAPDH

For eight samples, a PCR was setup against the housekeeping gene GAPDH to ensure that the samples contained enough canine DNA for later PCR of the SMOC2 gene. All eight samples generated a band of correct size of 108 bp on the agarose gel. It was concluded that the canine DNA concentration in the samples should be sufficient for further testing.

3.2 Dilution series

Since there was limited amount of DNA from each dog in the study, a dilution series was performed to check if the samples could be diluted so that less DNA could be used for each testing. Three of the eight samples described above was diluted to 1:10 and 1:100 and tested for the GAPDH gene. However, for one sample, the DNA concentration of DNA after diluting was probably too low to get amplified, resulting in no visible bands after gel electrophoresis. Consequently, it was decided not to dilute DNA samples from the buccal swabs for further testing.

3.3 SMOC2

By setting up a PCR, the genotypes for the SMOC2 gene was obtained. Amplification products were visualised on an agarose gel (figure 6), where the wild type allele is expected to generate a fragment of 531bp, and the mutated allele is expected to generate a fragment of 698 bp.

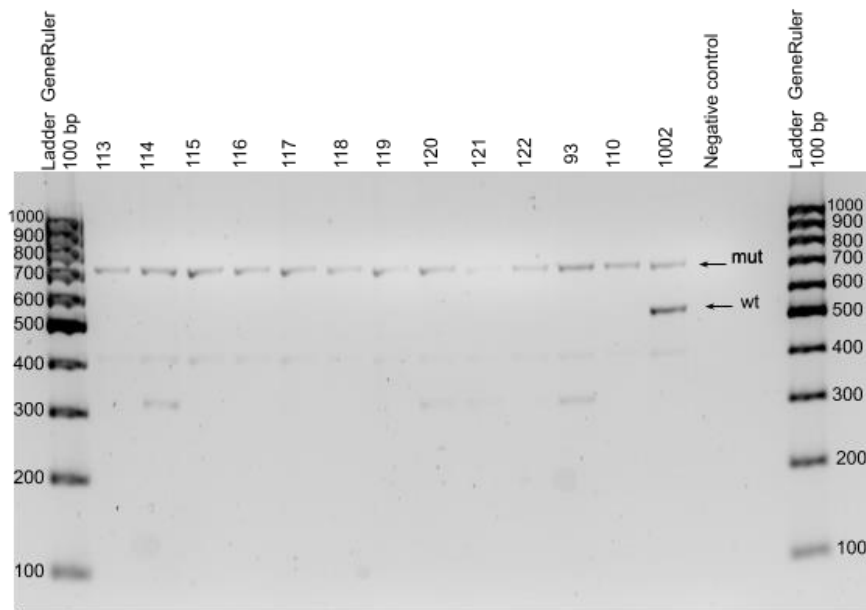


Figure 6. Agarose gel electrophoresis. Agarose gel electrophoresis showing SMOC2 PCR amplicons from different individuals. One band at about 700 bp indicates that the individual is homozygous for the mutation. Sample 1002, displaying two bands, is heterozygous for the mutation, carrying both the mutated LINE-1 insertion and the wild-type haplotype.

Table 3. *SMOC2* LINE-1 insertion.

Breed	Total genotyped dogs	WT	HET	MUT	Unknown
		SMOC2 ^{+/+}	SMOC2 ^{+/-}	SMOC2 ^{-/-}	
English Bulldog	18	0	0	15	3
French Bulldog	48	0	0	46	2
Boston Terrier	15	0	0	14	1
Pug	23*	0	0	23	0
Labrador Retriever	1	1	0	0	0
Golden Retriever	1	1	0	0	0
Chinese Crested Dog	2	1	1	0	0
Total	108	3	1	98	6

*Including DNA extracted from blood from two additional pugs, not participating in the inventory.

Out of 108 dogs genotyped for the SMOC2 mutation, 102 dogs (98 brachycephalic dogs and four non brachycephalic dogs) were successfully genotyped. All 98 successfully genotyped brachycephalic dogs were found to be homozygous for the mutation. As a control group, four non brachycephalic dogs were genotyped, consisting of one Labrador and one Golden Retriever and two Chinese crested dogs. One dog, a Chinese crested dog was found to be heterozygous for the SMOC2 mutation, the

other three dogs of the control group was genotyped as homozygous for the wild-type variant.

The allele frequency for the mutated allele when calculated for all brachycephalic dogs is 1.

3.4 Sanger Sequencing

By using Sanger sequencing, the genotypes for BMP3 and DVL2 for three dogs were obtained.

3.4.1 BMP3

The Golden Retriever was found homozygous for the ancestral C allele. Both the Pug and the English Bulldog was found homozygous for the derived A allele (Figure 7).

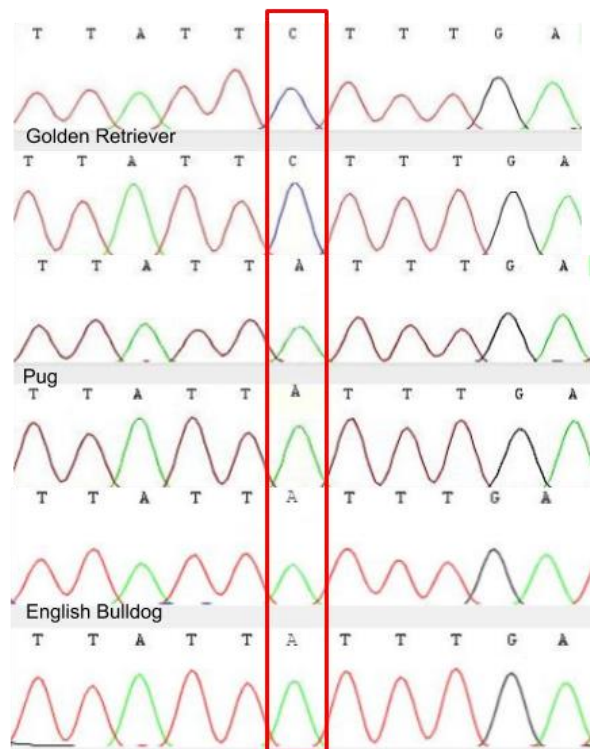


Figure 7. The figure shows the output for all three sampled dogs, Golden Retriever forward and reverse strand on top, Pug forward and reverse in the middle and English Bulldog forward and reverse bottom. The red box indicates the SNP position.

3.4.2 DVL2

The Golden Retriever and the Pug was found homozygous for the ancestral C allele. The English Bulldog was found homozygous for the deletion, causing the mutation.

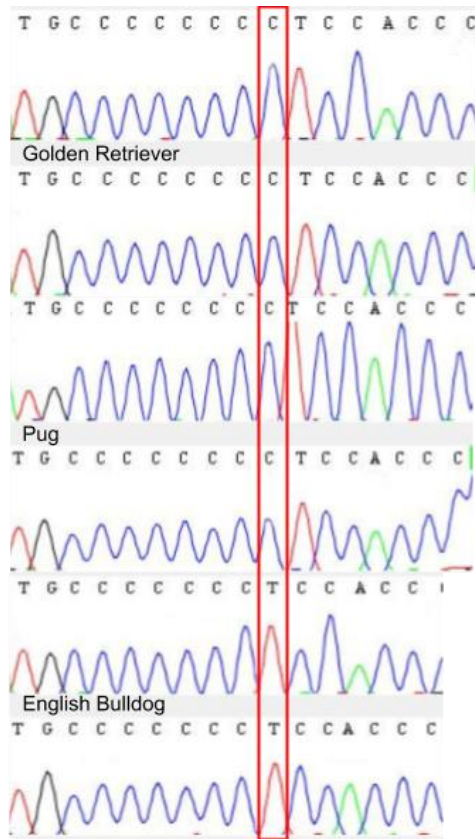


Figure 8. Visualisation of the SNP using Sanger Sequencing. The figure shows the output for all three sampled dogs. Golden Retriever front and reverse strand on top, Pug forward and reverse strand in the middle and English Bulldog forward and reverse strand bottom. The red box indicates the SNP position.

In summary, the English Bulldog was found homozygous for the mutations in both BMP3 and DVL2. The Pug was found homozygous for mutated variant of BMP3 and homozygous for the ancestral variant of DVL2. The non-brachycephalic Golden Retriever was homozygous for the ancestral variant in both studied genes (Table 4).

Table 4. Genotypes for BMP3 and DVL2 of three dogs.

Breed	BMP3	DVL2
English Bulldog	A/A	Del/del
Pug	A/A	C/C
Golden Retriever	C/C	C/C

3.5 BMP3

For BMP3, a TaqMan® SNP genotyping assays was performed to obtain the genotypes of 46 dogs (Figure 9). The C allele is presumed as the ancestral allele, called allele 1. The A allele is presumed as the derived (mutated) allele, called allele 2. Additionally, an artificial heterozygote sample was created by mixing DNA from the sequenced Golden Retriever, homozygous for allele A and the sequenced English Bulldog, homozygous for allele C. In total, 44 brachycephalic dogs were found homozygous for the A allele (Table 5). One English Bulldog was labelled as heterozygous, carrying one A allele and one C allele. The non-brachycephalic dog, a Golden Retriever was labelled as homozygous for the C allele. Also, the created artificial heterozygous sample was labelled as homozygous for the C allele.

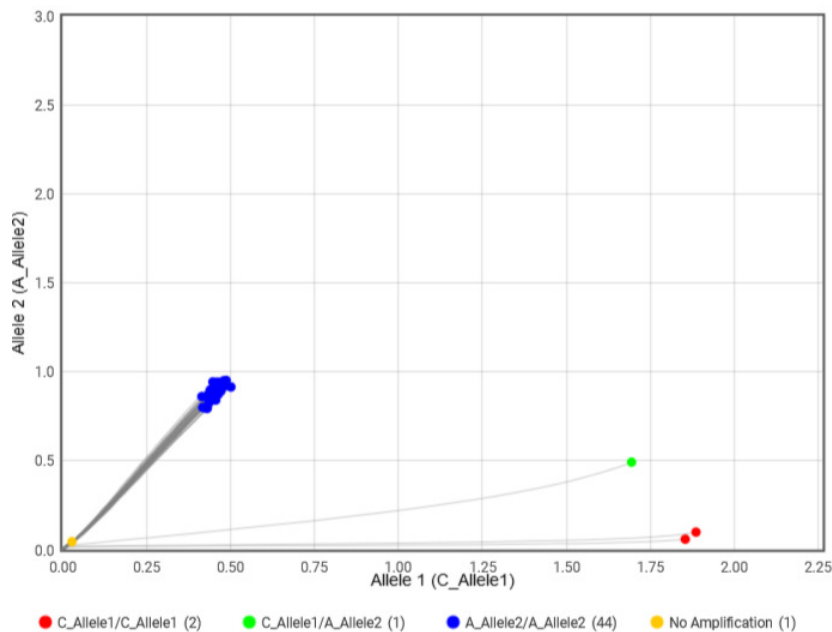


Figure 9. The two red dots in the right corner represents the samples found homozygous for allele A, the sequenced Golden Retriever and the artificial heterozygote. The green dot close to the middle represents the English Bulldog labelled as heterozygous. The blue upper cluster represents all other brachycephalic dogs, homozygous for the C allele.

Table 5. *BMP3* status variant across breeds

Breed	Total genotyped dogs	WT C/C	HET C/A	MUT A/A
English Bulldog	13	0	1	12
French Bulldog	9	0	0	9
Boston Terrier	13	0	0	13
Pug	10	0	0	10
Golden Retriever	1	1	0	0
Total	46	1	1	44

The allele frequency for the mutated allele when calculated for all four breeds together is 0.988.

3.6 DVL2

For DVL2, a TaqMan® SNP genotyping assays was performed to obtain the genotypes of 46 dogs (Figure 10). The C allele is presumed as the ancestral allele, called allele 1. The deletion causing the mutation is called allele 2. As described for the BMP3, an artificial heterozygous was created also for this genotyping assay. In total, 35 of the brachycephalic dogs was labelled as homozygous for the mutation. All ten genotyped pugs and the sequenced Golden Retriever was labelled as heterozygous. The artificial heterozygote sample is found just slightly above the lower cluster.

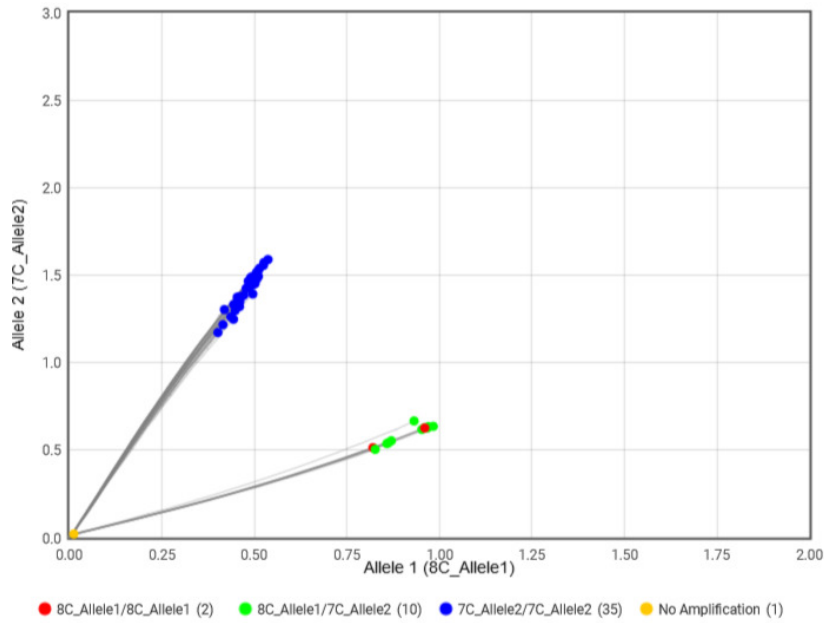


Figure 10. The two red dots in the lower green cluster in the plot represents the sequenced homozygous Golden Retriever and the sequenced homozygous Pug. All other green dots in the lower cluster is Pugs. The green dot slightly above the lower cluster represents the artificial heterozygous. The upper blue cluster represents all English Bulldogs, French Bulldogs and Boston Terriers.

Table 6. *DVL2* status (*DVL2c.2044delC*) variant across breeds

Breed	Total genotyped dogs	WT	HET	MUT
		C/C	C/del	Del/del
English Bulldog	13	0	0	13
French Bulldog	9	0	0	9
Boston Terrier	13	0	0	13
Pug	10	10	0	0
Golden Retriever	1	1	0	0
Total	46	11	0	35

Allele frequency for the mutated allele when calculated for all four breeds together is 0.75. When calculating the allele frequency for just the bull type breeds (Boston Terrier, English Bulldog and French Bulldog), the allele frequency for the mutated allele is 1.

4 Discussion and conclusions

The aim of this thesis was to investigate the genetic variation in known genes associated with brachycephaly in dogs. Three genes were studied, *SMOC2*, *BMP3* and *DVL2*. In total, 102 brachycephalic dogs were genotyped for the mutation in the *SMOC2* gene and 46 of these dogs were also genotyped for the mutations in the *BMP3* gene and *DVL2* gene respectively.

4.1.1 SMOC2

The *SMOC2* LINE-insertion is thought to highly influence the brachycephalic phenotype. Marchant et al. (2017) suggest that the size effects of the associated *SMOC2* haplotype accounts for 36 % of the facial length variation in the dogs in their study. In this thesis, all 102 successfully genotyped brachycephalic dogs were found to be homozygous for the *SMOC2* LINE-1 insertion. Consequently, the allele frequency for the derived allele was calculated to 1 in all studied breeds, suggesting that the mutation is or is close to fixation.

In a communication from Bannasch (2019), the allele frequency for the LINE-1 insertion was calculated to 0.97 for dogs of Bull type (Boston Terrier, Bulldog and French Bulldog) and 0.75 for Asian type breeds (Pug, Pekingese and Shih Tzu). The high allele frequency in the bull type breeds is similar when compared to the result in this thesis. For the Pugs, representing the Asia type breeds, the allele frequency in this study was higher since all individuals were found homozygous for the mutation.

In contrast to the study by Mansour et al. (2018), where three of the studied Boston Terriers was found heterozygous, all brachycephalic dogs were found homozygous in this thesis.

One interesting finding when genotyping dogs for the *SMOC2* mutation was that one of the Chinese crested dogs (non-brachycephalic breed) used as a positive control was found heterozygous, carrying one wild type allele and one mutated allele. The other Chinese crested, related to the first individual, was found homozygous for the wild type allele. Due to this, it would be interesting to sequence the heterozygous Chinese crested to confirm the genotype and later to genotype even more non-brachycephalic dogs, and especially Chinese crested dogs to better understand the mutation and investigate if heterozygous individuals occur in other non-brachycephalic breeds.

4.1.2 BMP3

BMP3 is thought to be required for craniofacial development in zebrafishes and the identified mutation in brachycephalic dogs is thought to be associated with skull shape.

DNA from three dogs in this thesis was sequenced, using the Sanger Sequence method, to determine the nucleotide sequence of the DNA covering the mutation. The sequenced Golden Retriever was found to be homozygous for the wild type allele and the two brachycephalic dogs (one English Bulldog and one Pug) was found homozygous for the mutated allele. The sequenced dogs were used as positive controls in the TaqMan genotyping assay, which was used to genotype 43 additional brachycephalic dogs. As a fourth positive control, there was an attempt of using an artificial heterozygous by mixing DNA from a homozygous wild type and homozygous mutated. However, the difference in DNA concentration between the two samples were not accounted for, which most likely lead to an overload of wild type DNA in the sample. Consequently, the artificial heterozygous was not recognized as heterozygous and positioned just slightly above the two homozygous wild types in the plot (figure 9).

Most of the brachycephalic dogs genotyped using the TaqMan assay clustered with the sequenced and confirmed homozygous mutated dogs. Therefore, it's a high possibility that also these dogs are homozygous mutated. The allele frequency for the mutated allele was 0.98, calculated for all dogs together. Communication from Bannasch (2019) suggests an allele frequency for the mutated allele at 0.99 in dogs of Bull type (Boston Terrier, Bulldog and French Bulldog) 0.99 and 0.89 in Asian type (Pug, Pekingese, Shih Tzu). The allele frequencies found in this study is consistent with these results, suggesting that the mutation in *BMP3* is or is close to fixation in brachycephalic dogs. However, one English Bulldog, was labelled as heterozygous for the *BMP3* mutation. It may be hard to draw any conclusions for

that sample. To determine if it truly is heterozygous or if errors or contamination have occurred would probably need to be confirmed by sequencing. Previous genotyped English Bulldogs in the literature have been found to be homozygous, thereof more work need to be done to correctly genotype the stated heterozygous Bulldog.

4.1.3 DVL2

The *DVL2* gene has been found to be a key component in the *Wnt* signalling pathway, important for several steps in the embryonic development. A rare syndrome in humans associated with craniofacial malformations, called Robinow syndrome, caused by mutations in the human *DVL1* and *DVL3* genes. The phenotype of people with Robinow syndrome have similar appearance to that of brachycephalic dogs, for example widely spaced eyes, wide forehead and a short nose. This has raised suspicions that the mutation in the canine *DVL2* gene causing the screw tail in brachycephalic breeds might also be involved in the shortened skull in such breeds (Mansour *et al.* 2018).

As mentioned above for the *BMP3* gene, the same three dogs were also sequenced using the Sanger sequencing method to determine the nucleotide sequence, covering the deletion in the *DVL2* gene. Two of the dogs, the Golden Retriever and the Pug was found homozygous for the wild type, while the English Bulldog was found to be homozygous for the mutation. The sequenced dogs were used as positive controls in the TaqMan genotyping assay, which was used to obtain the genotypes of additionally 43 brachycephalic dogs. For this genotyping assay there was also an attempt of using an artificial heterozygous. Like described in the section above, it is most likely that the higher concentration influenced the genotyping, resulting in that the artificial heterozygous was positioned just slightly in the lower cluster seen in plot (Figure 10).

In total, 46 dogs were genotyped. Dogs of bull type (e.g. Boston Terrier, English Bulldog and French Bulldog) was clustered as homozygous mutated, in the same cluster as the English Bulldog confirmed as homozygous mutated after sequencing. All genotyped Asian type dogs (e.g. Pugs) are visible in a second lower cluster in the allelic discrimination plot (green lower cluster in figure 10). The algorithm labels samples in this cluster as heterozygous, though, the two dogs confirmed as homozygous wild type (the Golden Retriever and the Pug) is also positioned in this cluster. Therefore, it is more likely that samples in this cluster carries two wild type alleles, which is not recognised in the software for unknown reasons. The study by Mansour *et al.* (2018) also supports that Pugs, as a breed lacking screw tails, are

homozygous for the wild type allele. It is possible that the assay or the protocol needs further optimisation to correctly label wild type samples.

The allele frequency for the mutated allele is calculated to 0.75 when including all four breeds. When calculating the allele frequency only including Bull type, the allele frequency for the mutated allele was found to be 0.94. Since all Pugs was found homozygous wild type, it may be more correct to exclude Pugs when calculating allele frequency. Bannasch (2019) suggest an allele frequency of 0.98 in dogs of Bull type and an allele frequency of 0.01 in dogs of Asia type, consistent with the results in this thesis. This suggests that also the mutation in the *DVL2* gene is or is close to fixation in the studied population of Boston Terrier, English Bulldog and French Bulldog.

Studied Pugs were found to be homozygous for the ancestral type allele of *DVL2*, still, Pugs are very much brachycephalic. The mutation has been associated with the screw tail, characteristic for breeds such as Boston Terrier, English Bulldog and French Bulldog. (Mansour *et al.* 2018). However, if and in that case how the *DVL2* gene is involved in the craniofacial development and if the found mutation can be associated with the shortened skull in brachycephalic breeds needs to be studied further.

4.2 Strengths and weaknesses with this thesis

One strength with this study is that even though it can be considered as a rather small study, 102 dogs were successfully genotyped for the *SMOC2* LINE-1 insertion, which is a rather high number of dogs in relation to the size of the project. In previous studies of the *SMOC2* mutation, 374 (37 brachycephalic) and 152 (109 brachycephalic) dogs were genotyped, respectively (Marchant *et al.* 2017; Mansour *et al.* 2018).

However, one weakness with this study was that only four dogs of non-brachycephalic breeds was used as positive controls when studying the three genes. Previous studies of described genes have studied and genotyped dogs from several breeds, both brachycephalic and non-brachycephalic, which can be considered as a strength. For future research, more dogs of different breeds should be included.

Another strength with this thesis is that the found allele frequencies for the three genes are all consistent with the frequencies in previous performed studies.

Unfortunately, due to lack of time, not all dogs in the study was genotyped for all three genes which was an ambition in this thesis. Only less than half of the dogs were genotyped for the mutations in *BMP3* and *DVL2* which can be considered as a weakness. For future studies, it would be interesting to include dogs not genotyped in this study. Also, the amplification was found very low when using the TaqMan genotyping assays, which can be seen in the allelic discrimination plots (Figure 9 and 10). That the assay did not work optimal is a weakness in this thesis. It is possible that the assay needs further optimization to get a higher amplification before potential future research.

One opportunity for future work would be to estimate the total additive genetic variation in the breeds, based on phenotype and pedigree data. However, the population size in this thesis was too small to estimate genetic variation and heritabilities.

4.3 Relationship in the studied population

To investigate the population structure and to see whether the studied population was representative for the whole Swedish population, pedigree data was used to study the relatedness in and between the two populations. The statistical software SAS 9.4 was used to merge data from the inventories with pedigree data provided by the Swedish kennel Club by the registration number of participating dogs. The dogs that were part of the inventory and this thesis was set as group 1, dogs in the pedigree file born in or after the year 2000 was set as group 2. The contribution, inbreeding (F), coancestry (CFC) software (Sargolzaei *et al.* 2006) was used to estimate average numerator relationships between the two groups.

Table 7. Average relationship in and between groups

Breed	Dogs in group 1	Dogs in group 2	Relationship group 1	Relationship group 2	Relationship between group 1 and group 2
Boston Terrier	15	2600	0.10	0.02	0.02
English Bulldog	17	3146	0.08	0.01	0.01
French Bulldog	41	10361	0.046	0.01	0.01
Pug	20	9385	0.099	0.028	0.03

For all four breeds, the dogs in group 1 was estimated to be more related to each other than to the dogs in group 2. For future studies, it might however be of interest to study dogs less related that would better represent the bigger population.

4.4 Phenotypic variation

Bertilsson (2019) investigated the phenotypic variance correlated to BOAS in 119 brachycephalic dogs collected during the inventories described in this thesis. Some phenotypic variation was found in the four breeds; Boston Terrier, English Bulldog, French Bulldog and Pug (Table 8). However, only English Bulldog and French Bulldog was suggested to have enough phenotypic variation to improve the BOAS problem. The Boston Terrier population was too small and the BOAS problem was not severe enough to get any results. For Pugs, the phenotypic variance was found to be very small and a need of a more drastic approach to handle the BOAS problems was suggested.

Table 8. Phenotypic variance and mean value for measurements and ratios

		Boston Terrier	English Bulldog	French Bulldog	Pug
Snout length (mm)	Variance	20-30	15-46	19-38	11-25
	Mean	23.5	32.8	29.3	17
Cranial length (mm)	Variance	80-125	125-185	95-170	90-150
	Mean	105.7	150.1	121.3	111.5
CFR	Variance	0.16-0.31	0.12	0.15-0.39	0.09-0.22
	Mean	0.23	0.22	0.24	0.16

Adapted from Bertilsson (2019), with permission.

As can be seen in Table 8 and the master's thesis by Bertilsson, there is some phenotypic variation in the four breeds. Dogs not affected by BOAS were also shown to have significantly longer snouts, longer sternum and higher CRF, compared to BOAS affected dogs. Non affected dogs also had a significantly larger variance for snout length, chest girth, sternum length, back length, CFR and NGR, compared to affected dogs (Bertilsson, 2019).

The thesis by Bertilsson suggests that there is some phenotypic variation that can be observed in the studied dogs. On the contrary, the genetic variation in the studied genes associated with brachycephaly is suggested as low. Marchant et al. (2017) suggests that homozygous-derived alleles of the *SMOC2* LINE-1 solely explains 36 % of the viscerocranium variation while *BMP3* is thought to explain 12 % of the variation. It should be noted that the variances are not additive but implies the

maximum potential proportion of each genotype. It is likely that the brachycephalic phenotype is complex trait, affected by many genes, both known and probably yet unknown genes. Most likely, more dogs need to be studied and genotyped to get a better picture of genes associated with brachycephaly and to further investigate the genetic variation in the populations. For the Swedish project, about 50-100 dogs per breed will be genotyped using Illumina CanineHD Whole-Genome Genotyping BeadChip (170K) to identify genomic regions associated with increased risk for BOAS. For future genotyping, it would be of interest to base the selection on pedigree data to include animals not related to each other.

4.5 Conclusions

In this master thesis, 102 privately owned brachycephalic dogs was genotyped for the mutation in the *SMOC2* gene, additionally, 45 of these dogs were also genotyped for the mutations in the *BMP3* and *DVL2* gene. The results in this thesis indicates that there is no or very little variation in the three genes and that the mutations in previous mentioned genes is or is close to fixation in the studied population. The mutant variant of the *SMOC2* gene was found to be fixated in the studied four breeds. The mutant variant of the *BMP3* gene had high allele frequency in all breeds. In Bull type breeds (Boston Terrier, English Bulldog and French Bulldog) the mutant version of the *DVL2* gene was fixated, whilst all Pugs were tested wild type for the *DVL2* mutation. However, the studied population is too small to draw any conclusion and further research is needed to thoroughly investigate the genetic variation in the four breeds.

5 Possible future scenarios for the brachycephalic breeds

As suggested, more research is needed to genotype more dogs in order to get a better picture of the genetic variation in Swedish brachycephalic dogs. The following part of the will therefore discuss two possible scenarios after further future research.

5.1.1 Enough genetic variation

Future studies of the brachycephalic dog populations may conclude that the genetic variation in brachycephalic breeds is not lower than the genetic variation observed in other dog breeds. If it is deemed possible to genetically select dogs within breed for a conformation less associated with welfare problems, there might be a need to implement assessment tools in the breeding plans to reduce the health issues observed in brachycephalic dogs.

Today, the Swedish kennel club have several ongoing projects to decrease health issues in brachycephalic breeds and how to come to terms with extreme conformations causing respiratory problems. One of these projects is a new puppy examination protocol that was introduced in 2017, after an uprising where hundreds of Swedish veterinarians was demanding the Swedish kennel club and Swedish board of Agriculture to come to terms with breeding and import of dogs with respiratory problems. The examination protocol is to be used for all registered puppies up to six months of age and can also be used for unregistered puppies. The purpose of the examination protocol is to early catch dogs with respiratory problems (Svenska kennelklubben 2017).

An examination before breeding of brachycephalic dogs has also been tried out and accepted. During the examination, the dog is examined during rest and after exercise, where the dog is supposed to trot 1 000 meters in less than 12 minutes. The examination is intended to work as a tool to assess symptoms of respiratory problems and difficulties to thermoregulate during rest and exercise. The conformation and the possible contributions to health issues associated with brachycephaly of the dog and its offspring is also assessed. In February 2019, the examination was accepted as a health program on level 1, which means it is not mandatory but the results from the examination will be registered at the Swedish kennel club and displayed at the SKK breeding data online. In the future, the health program will be evaluated and might be transformed to a health program on level 2 (requirements on performed examination) or at level 3 (requirements on performed examination with demands of certain result). The examination can be performed at two years of age and the dog can redo the examination after six months (Svenska kennelklubben 2019).

The Swedish kennel club has also suggested that surgical procedures on dogs of brachycephalic type and/or death or euthanasia due to problems associated to brachycephaly should be registered at the kennel club. Veterinary clinics should handle the reporting to the kennel club and dogs that have gone through surgery should not be used for breeding (Svenska kennelklubben).

Additionally, the Swedish kennel club is stating that healthy dogs with less extreme conformation should be prioritized and placed at dog shows. Breed specific instructions (BSI) have therefore been used since 2009 in Sweden and since 2014 there is a Nordic collaboration against extreme conformation with the help of a common BSI, which should be used as a complement to the breed standards. The breed specific instructions identify areas of risk and aims at preventing possible future problems. BSI includes both instructions concerning all dogs, and instructions only concerning specific “breed types”. The BSI states that “*All dogs should be able to breathe normally, also when moving*” (The Nordic Kennel Club, 2018, p 10). The statement above regards all dogs, in addition, brachycephalic breed type also has instructions implying that exaggerations in typical features such as skull, muzzle, jaw, eyes, ribcage and skin might lead to serious health problems and not exclusively problems in breathing and thermoregulation. The breathing distress should be assessed in the show ring. Nonsignificant affected breathing should be noted but not necessarily affecting the quality grading of the dog. Milder respiratory problems for example pinched nostrils and too short nose should influence the quality grading. Obvious signs of respiratory problems should motivate for disqualification (The Nordic Kennel Club 2018). From 2020 to 2024 only Nordic show judges will be allowed to judge brachycephalic breeds. Further, the Swedish kennel club is actively working

to educate and inform dog show judges about health problems associated with extreme conformations.

As stated above, the Swedish kennel club has taken many actions to reduce health problems in brachycephalic breeds by several selection criterions. The problems with brachycephalic dogs will need to be handled by a united mass of both breeders, dog owners, show judges, veterinarians and the kennel club. This can of course be problematic, but the work led by the kennel club is a step in the right direction. The current suggested actions to obtain brachycephalic dogs with less extreme phenotypes and better health would need to be evaluated before taking discussing further actions.

The health of brachycephalic dogs is a hot topic, not only in Sweden. In February 2019, the Dutch animal welfare legislation published a report with changes the animal welfare legislation, mainly regarding breeding of brachycephalic breeds. The workgroup suggests a so-called “traffic light model” to be used as a tool to easier select for dogs with less extreme conformation and a better health. Each criterium in the suggestion has a traffic light coding; green, orange or red. In total, 28 criterions are listed in the report, divided into categories such as traits associated with BOAS and Brachycephalic Ocular syndrome (BOS). Examples from the traffic light model is described in table 9. It’s suggested that dogs that are categorised as green can be used for breeding, dogs with one orange “light” can be used for breeding if all other criteria are green. Dogs categorised as red are not allowed to be used for breeding. The suggestion is to allow dogs categorised as orange for a shorter period, for example for two generations and later only allow “green” breeding animal. The stepwise motion of the suggestion is to not reduce the breeding population drastically. Breeders have been given a time to breed toward a healthier type, if this don’t occur, a mandatory breeding program will be discussed.

Table 9. Examples of criterium listed in Dutch animal welfare legislation

Criterion	Green (ideal)	Orange	Red	Consequence if not ideal
Nostrils	Open nostrils	Mild stenosis	Severe stenosis	Orange may be used if no other criteria is exceeded
CFR	≥ 0.5	0.3-0.5	≤ 0.3	Orange may be used if no other criteria is exceeded

Criterion	Green (ideal)	Orange	Red	Consequence if not ideal
Abnormal respiratory noise	Absent	-	Strong sniffing, snoring or noisy breathing at rest	Exceeding standards, regardless of other criteria
6 minutes test	>500 m, restored heart frequency and body temp within 5 min	>500 m, restored heart frequency and body temp within 10 min	<500 m, heart frequency and body temp not restored within 10 min	Orange may be used if no other criteria is exceeded
1000 m test	Maximum 12 min			

Table adapted from van Hagen (2019).

The Dutch report highlights many criteria associated with brachycephaly and BOAS. There are similarities between the work led by The Swedish kennel club and the Dutch animal welfare legislations, for example that breeding animals should show good respiration and good thermoregulation, both in rest and during/after exercise. The Dutch legislation can however be considered as more drastic when involving criteria regarding measures and ratios. The report refers to many scientific articles concerning brachycephaly and/or BOAS but the exact reasoning for using the stated measures and ratios stated is not clear.

By looking at the studied Swedish population of brachycephalic dogs, it would be hard or perhaps not even possible to incorporate such criteria on measures and ratios to the breeding programs. The Dutch legislation states that a dog with a CFR under 0.3 should not be used for breeding. A dog with a CFR between 0.3 and 0.5 should only be used if all other criteria are fulfilled. For the studied population, the mean values for CFR is 0.22, 0.21, 0.24 and 0.15 for Boston Terrier, English Bulldog, French Bulldog and Pug respectively. For the three bull-type breeds, there would only be a small number of dogs fulfilling the CFR criterion, provided that the dog fulfils all other criteria. No pug in this studied population would fulfil the CFR criterion, according to the Dutch legislation. Implying such criteria on the Swedish brachycephalic dog population would probably drastically decrease the effective population size. The health program, the new puppy examination protocol and the focus on awarding healthy and less “extreme” dogs at dog shows are selection criteria provided by the Swedish kennel club today and the consequences of using these tools should be evaluated before taking more drastic solutions under consideration.

Breeders and keepers should carefully evaluate future breeding animals to ensure that dogs with a healthy conformation is used for breeding.

If the phenotypic variation and heritabilities is shown to be high enough for traits associated with BOAS, there might be possible to implement traditional estimated breeding values (EBV). Most conformation traits have been found to have quite high heritabilities. For example, in horses where body length has shown to have an estimated heritability of 0.72. The head length had an estimated heritability of 0.55 (Mcmanus *et al.* 2002).

EBVs have been used for hip and elbow dysplasia, where abnormal formation of the joint can cause pain, are complex traits that have used estimated breeding values to come to terms with the problems. Breeding values for hip- and elbow dysplasia is estimated by using so called Best Linear Unbiased Prediction (BLUP), which uses both information from the animal, its offsprings and from relatives, using pedigree data to estimate breeding values. BLUP is widely used for several animal categories, including horses. However, BLUP requires reliable pedigrees and systematically recording of phenotypes. To be able to use BLUP for brachycephalic dogs, measurements of conformations traits would need to be recorded for a high number of dogs. One suggestion could be to collect such phenotype data at the same time as earlier mentioned evaluation for breeding.

Recently, there has also been several research projects to investigate the possibilities to use genomic selection for hip and elbow dysplasia. Sánchez-Melano *et al.* (2015) found that the accuracy was highest when combining pedigree and genomic data, compared to traditional breeding evaluation (Sánchez-Molano *et al.* 2015). For the future, it would perhaps be a possibility to use genomic selection to come to term with health problems associated with canine brachycephaly.

5.1.2 Not enough genetic variation in the breeds

With low genetic variation, it will be difficult to select for traits to decrease extreme conformations and health problems in the brachycephalic breeds. To improve the overall health of brachycephalic dogs, outcrossing may be a future option. For a small number of breeds in Sweden outcrossing projects have been implemented in the breeding strategies.

In 2001 the Clumber Spaniel club applied for outcrossing to increase the genetic variation in the breed. For the outcrossing, a Cocker Spaniel of hunting type was selected since the Cocker Spaniel is closely related to the Clumber, is a healthy and sound breed without extreme conformation and without diseases not present in the Clumber breed. In 2003 the first outcrossed litter was born. From that first litter, two

females were backcrossed with Clumber Spaniel males. Two females from the backcross litter was later accepted and registered as purebred Clumber Spaniels. The health of the dogs in the outcrossing project is similar to the health of the whole Clumber Spaniel population, except occurrence of elbow dysplasia that was slightly higher for the first outcross generation. The mentality of the latest generation in the outcrossing project is not deviating from the rest of the Clumber population (Clumber Spaniel Klubben 2014).

Another breed with an ongoing outcrossing project is the Norwegian Lundehund. The Lundehund has been close to extinction multiple times, for example in the 60's where the breed consisted of just six known individuals. After the multiple genetic bottle necks, the genetic variation in the breed was found low. The Norwegian Lundehund Club therefor applied for an outcrossing project, which was accepted by the Norwegian kennel club. The outcrossing projects aim is to increase the genetic variation in the breed and by that hopefully increase fertility and decrease the occurrence the disease Intestinal lymphangiectasias currently present in the breed. Related breeds such as the Norwegian Buhund, Norrbotten-Spitz and the Icelandic Sheepdog has been selected for the outcrossing project to form a side population registered in a so-called X-register. Females of these three breeds can be mated with a Lundehund male to generate the first generation of crosses (F1), females from the F1 generation can later be backcrossed with Lundehund to generate a F2 generation. Health, exterior and mentality will be tested for later generations before crosses in the X-register can be allowed in to the Lundehund breed (Fossen 2019).

If an outbreeding project would be in question for brachycephalic breeds, there could be some problematic parts that would have to be handled:

- What breed or breeds could be suitable for cross breeding?
- What would be the criteria for such possible breeds?
- Are breeders and dog owners willing to participate in such projects?
- Which criteria would need to be fulfilled for including crossbreds in the purebred population?
- Will future crossbred individuals be accepted, and would they be used for breeding?

If it will come to a point where an outbreeding project would be the solution, joined efforts from breeders, the kennel club and dog owners will be needed.

5.1.3 Breed standards and breed-specific breeding instructions

A suggestion is to investigate if there is a need to clarify and improve breed standards for the four studied breeds. All four breed standards state the muzzle should be short, relatively short or very short (FCI-standard N° 149, 2011; FCI-standard N°253, 2010; FCI-standard N°101, 2015). The standard for the Boston Terrier states that the length of the muzzle should not be longer than one third of the length of the head (FCI-standard N° 140, 2014). Breed standards, breeders and dog show judges have possibilities to together improve the health of brachycephalic dogs.

All breeds have breed-specific breeding instructions (RAS) that describes problems and strong aspects within the breed and breeding recommendations. All breeding recommendations mentions that dogs used for breeding should be able to breathe normally. The breeding recommendations for Boston Terrier recommends not to breed on dogs with respiratory problems. The recommendation also states that the breed today do not have any conformation problems (Svenska Bostonterrierklubben 2011).

The recommendations for the French Bulldog recommend that dogs with extreme conformation should not be used for breeding. Further, dogs with stenotic nostrils, elongated soft palate, narrowing of or problems in the respiratory tract should not be used for breeding. (Fransk Bulldogg klubb 2010)

The English Bulldog recommendations states that normal breathing function should be of highest priority when selecting breeding animals. Sufficient length of muzzle and other structures affecting breathing should be a selection criterion (Projektgruppen för engelsk bulldogg 2015).

For the Pug, dogs with normal breathing function and less nose wrinkled should be prioritized. Dogs with stenotic nostrils, elongated soft palate or narrowing of or problems in the larynx or throat should not be used for breeding (Mopsorden 2015).

The mentioned recommendations are a positive step in the right direction. It may however be necessary to investigate how well the recommendations are applied in the breeding work.

5.2 Weaknesses in study and future perspectives

This part of the thesis will concern steps that did not work as good as predicted. Maybe, it will be helpful for other students or scientist struggling.

Buccal cells were sampled using 2-3 cytology brushes per dog, simply by stroking the inside of the dog's cheek with the brush. This method is very simple, not invasive for the animal and can be performed by anyone by simple instructions. The cytology brushes were airdried before putting them in a marked paper envelope and sealed. The envelopes were later stored in room temperature before performing the DNA extraction. It's hard to say if this was the best way of storing the brushes, or if it would be better to perform the extraction closer to the sampling. Most swab kits require that the swab is put in a stabilizing buffer to prevent fragmentation of the DNA. However, if this would improve the amplification is hard to say. Also, since many bacteria's can be present in a dog's mouth, the measurements of nucleic acid got problematic since there was no way to tell how much of the nucleic acid that was canine and how much that was bacterial. The variation in DNA concentration in the samples were problematic when performing a dilution series, therefor, samples from swabs was not diluted.

For the SMOC2 PCR, the standard protocol for the HotStar® polymerase using suitable annealing temperature did not work optimal, resulting in many unspecific bands could visible after gel electrophoresis. Also, optimising by performing a series of different annealing temperatures did just slightly improve the specificity. After communication with Marchant, T., the corresponding author of the SMOC2 article, a working and optimized protocol was received. By using this protocol, fragments of expect size was amplified, but still there was some unspecific amplicons visualised after gel electrophoresis. Attempts to optimize the PCR conditions by optimizing the Primer concentrations, dNTP concentration and the magnesium concentration did increase the primer specificity, after gel electrophoresis there were still unspecific bands showing. It should be mentioned that the amplification worked well enough to determine the genotypes of the dogs, but to get rid of unspecific bands further optimization is probably needed. One suggestion on optimization is to use the same polymerase as described my Marchant, T (personal communication, 2019) and to add SDS when performing gel electrophoresis to increase the specificity.

Another problem for this PCR was that not all samples was amplified correctly, leaving the genotype undetermined. This is most likely due to low amount of DNA in the samples or possibly due to fragmentation or inhibition of the DNA in the swab samples.

For the TaqMan genotyping assays, the first qPCR was run without any positive controls since no dog had a known genotype from start. Consequently, all samples were labelled as undetermined by the StepOne™ software. The amplification was also, as mentioned earlier, quite low. Due to this result, it was decided to sequence three samples using Sanger sequencing to obtained dogs with a confirmed genotype and to use these as positive controls. The sequencing step was not planned for and took extra time, resulting in not having enough time to genotype all samples using the TaqMan assay. As mentioned for the SMOC2 PCR the different DNA concentrations in the samples might have contributed to the problems with the TaqMan assays. The assays are sensitive and requires a certain amount of DNA to generate correct assignment. For both assays, the amplification remained low, even after adding the positive controls and optimizing the annealing temperature for each primer. For *DVL2*, dogs confirmed as homozygous wildtype was labelled as heterozygous. Due to lack of time, this result could not be investigated further but would probably need to be sorted out before continuing with future research. Possibilities for these kinds of errors could be the lack of standardisation of DNA concentration or additional SNPs in the target area, affecting the efficiency of the amplification. Since there are difficulties determining the concentration of canine DNA when sampled from swabs, one alternative could perhaps be to use DNA sampled from blood to easier measure the DNA concentration and use correct DNA amount for the assays. Blood samples may however be more stressful for the dog and would require trained personnel, which should be considered before deciding on changing sample method.

For future perspectives, it would be of high importance and interest to investigate more dogs and in particular, more genes found associated with the brachycephalic phenotype. There is ongoing research in the field and success in such research should be observed and if suitable applied on the Swedish population. Only three of known genes associated with brachycephaly is covered in this thesis, recent research has for example studied genes such as *FGF*, *P2RX7*, *STC2* and a QTL on chromosome 30. A recently published article discovered missense mutation in the gene *ADAMTS3* on chromosome 13. The mutation was associated with a respiratory disease syndrome in the Norwich Terrier, same as can be seen in the Bulldog breed. The study screened a large number of dogs for the mutation and suggests that the found variant may influence BOAS in French and English Bulldogs. It would be of high interest to also screen the Swedish population of brachycephalic dogs for the

ADAMTS3 mutation for further investigation and explore the possibilities to use such mutations as a diagnostic marker for disease.

References

- American Kennel Club *Bulldog history (Origin of the English bulldog)*. Available at: <http://www.bulldoginformation.com/english-bulldog-history.html> [2019-02-25]
- American kennel club *Pug Dog Breed Information*. American Kennel Club. Available at: <https://www.akc.org/dog-breeds/pug/> [2019-03-18]
- Berilsson, I. (2019) *Phenotypic variation of BOAS within four brachycephalic dog breeds - Can good welfare be obtained?* Unpublished manuscript. The Swedish University of Agricultural Sciences. Uppsala.
- Bill, B.R., Petzold, A.M., Clark, K.J., Schimmenti, L.A. & Ekker, S.C. (2009). A Primer for Morpholino Use in Zebrafish. *Zebrafish*, vol. 6 (1), pp. 69–77
- Bloch-Zupan, A., Jamet, X., Etard, C., Laugel, V., Muller, J., Geoffroy, V., Strauss, J.-P., Pelletier, V., Marion, V., Poch, O., Strahle, U., Stoetzel, C. & Dollfus, H. (2011). Homozygosity Mapping and Candidate Prioritization Identify Mutations, Missed by Whole-Exome Sequencing, in SMOX2, Causing Major Dental Developmental Defects. *The American Journal of Human Genetics*, vol. 89 (6), pp. 773–781
- Bornstein, P. (2009). Matricellular proteins: an overview. *Journal of Cell Communication and Signaling*, vol. 3 (3), p. 163
- Boulet, A.M., Moon, A.M., Arenkiel, B.R. & Capecchi, M.R. (2004). The roles of Fgf4 and Fgf8 in limb bud initiation and outgrowth. *Developmental Biology*, vol. 273 (2), pp. 361–372
- Boutros, M. & Mlodzik, M. (1999). Dishevelled: at the crossroads of divergent intracellular signaling pathways. *Mechanisms of Development*, vol. 83 (1–2), pp. 27–37
- Boyko, A.R., Quignon, P., Li, L., Schoenebeck, J.J., Degenhardt, J.D., Lohmueller, K.E., Zhao, K., Brisbin, A., Parker, H.G., vonHoldt, B.M., Cargill, M., Auton, A., Reynolds, A., Elkhoulou, A.G., Castelano, M., Mosher, D.S., Sutter, N.B., Johnson, G.S., Novembre, J., Hubisz, M.J., Siepel, A., Wayne, R.K., Bustamante, C.D. & Ostrander, E.A. (2010). A Simple Genetic Architecture Underlies Morphological Variation in Dogs. *PLOS Biology*, vol. 8 (8), p. e1000451
- Cadigan, K.M. & Nusse, R. (1997). Wnt signaling: a common theme in animal development. *Genes & Development*, vol. 11 (24), pp. 3286–3305

- Chen, D., Zhao, M. & Mundy, G.R. (2004). Bone Morphogenetic Proteins. *Growth Factors*, vol. 22 (4), pp. 233–241
- Clumber Spaniel Klubben (2014). Rasspecifik Avelsstrategi för Clumber Spaniel. p. 20
- Daluiski, A., Engstrand, T., Bahamonde, M.E., Gamer, L.W., Agius, E., Stevenson, S.L., Cox, K., Rosen, V. & Lyons, K.M. (2001). Bone morphogenetic protein-3 is a negative regulator of bone density. *Nature Genetics*, vol. 27 (1), pp. 84–88
- Davis, M.S., Cummings, S.L. & Payton, M.E. (2017). Effect of brachycephaly and body condition score on respiratory thermoregulation of healthy dogs. *Journal of the American Veterinary Medical Association*, vol. 251 (10), pp. 1160–1165
- Evans, H.E. & Miller, M.E. (2013). *Miller's anatomy of the dog*. Fourth edition. St. Louis, Missouri: Elsevier.
- Farrell, L.L., Schoenebeck, J.J., Wiener, P., Clements, D.N. & Summers, K.M. (2015). The challenges of pedigree dog health: approaches to combating inherited disease. *Canine Genetics and Epidemiology*, vol. 2 (1), p. 3
- Fawcett, A., Barrs, V., Awad, M., Child, G., Brunel, L., Mooney, E., Martinez-Taboada, F., McDonald, B. & McGreevy, P. (2019). Consequences and Management of Canine Brachycephaly in Veterinary Practice: Perspectives from Australian Veterinarians and Veterinary Specialists. *Animals*, vol. 9 (3). DOI: <https://doi.org/10.3390/ani9010003>
- Feng, T., McConnell, C., O'Hara, K., Chai, J. & Spadafori, G. (2017). *Brachycephalic Breed Disease Prevalence Study*. Nationwide. Available at: <http://nationwidedvm.com/wp-content/uploads/2017/03/NWBrachycephalicStudy0317.pdf> [2019-03-05]
- Feng, W., Leach, S.M., Tipney, H., Phang, T., Geraci, M., Spritz, R.A., Hunter, L.E. & Williams, T. (2009). Spatial and Temporal Analysis of Gene Expression during Growth and Fusion of the Mouse Facial Prominences. (Bridger, J. M., ed.) *PLoS ONE*, vol. 4 (12), p. e8066
- Fossen, R. (2019-04-09). *Prosjektets bakgrunn*. *Norsk Lundehund Klubb*. Available at: <http://lundehund.no/index.php/krysningsprosjektet1/artikler-om-krysningsprosjektet/896-prosjektets-bakgrunn> [2019-05-15]
- Fransk Bulldogg klubb (2010). Avelspolicy och Avelsstrategi för Fransk Bulldogg. Svenska Kennelklubben. Available at: <https://www.skk.se/globalassets/dokument/utstallning/breed-special-specific-instructions---bsi-a8.pdf> [2019-05-16]
- Frantz, L.A.F., Mullin, V.E., Pionnier-Capitan, M., Lebrasseur, O., Ollivier, M., Perri, A., Linderholm, A., Mattiangeli, V., Teasdale, M.D., Dimopoulos, E.A., Tresset, A., Duffraisie, M., McCormick, F., Bartosiewicz, L., Gál, E., Nyerges, É.A., Sablin, M.V., Bréhard, S., Mashkour, M., Bălăşescu, A., Gillet, B., Hughes, S., Chassaing, O., Hitte, C., Vigne, J.-D., Dobney, K., Hänni, C., Bradley, D.G. & Larson, G. (2016). Genomic and archaeological evidence suggest a dual origin of domestic dogs. *Science*, vol. 352 (6290), pp. 1228–1231
- Freedman, A.H., Gronau, I., Schweizer, R.M., Vecchy, D.O.-D., Han, E., Silva, P.M., Galaverni, M., Fan, Z., Marx, P., Lorente-Galdos, B., Beale, H.,

- Ramirez, O., Hormozdiari, F., Alkan, C., Vilà, C., Squire, K., Geffen, E., Kusak, J., Boyko, A.R., Parker, H.G., Lee, C., Tadisotla, V., Siepel, A., Bustamante, C.D., Harkins, T.T., Nelson, S.F., Ostrander, E.A., Marques-Bonet, T., Wayne, R.K. & Novembre, J. (2014). Genome Sequencing Highlights the Dynamic Early History of Dogs. *PLOS Genetics*, vol. 10 (1), p. e1004016
- Freedman, A.H. & Wayne, R.K. (2017). Deciphering the Origin of Dogs: From Fossils to Genomes. *Annual Review of Animal Biosciences*, vol. 5 (1), pp. 281–307
- Gamer, L.W., Nove, J., Levin, M. & Rosen, V. (2005). BMP-3 is a novel inhibitor of both activin and BMP-4 signaling in *Xenopus* embryos. *Developmental Biology*, vol. 285 (1), pp. 156–168
- Gammons, M.V., Rutherford, T.J., Steinhart, Z., Angers, S. & Bienz, M. (2016). Essential role of the Dishevelled DEP domain in a Wnt-dependent human-cell-based complementation assay. *Journal of Cell Science*, vol. 129 (20), pp. 3892–3902
- Genetics Home Reference (2019). *Robinow syndrome*. *Genetics Home Reference*. Available at: <https://ghr.nlm.nih.gov/condition/robinow-syndrome> [2019-02-25]
- Gilbert, S.F. & Barresi, M.J.F. (2016). *Developmental biology*. Eleventh edition. Sunderland, Massachusetts: Sinauer Associates, Inc.
- van Hagen, M.A. (2019). *FOKKEN MET KORTSNUITIGE HONDEN Criteria ter handhaving van art. 3.4. Besluit Houders van dieren Fokken met Gezelschapsdieren*. Departement Dier in Wetenschap en Maatschappij en het Expertisecentrum Genetica Gezelschapsdieren.
- Harvey, C.E. (1989). Inherited and congenital airway conditions. *Journal of Small Animal Practice*, vol. 30 (3), pp. 184–187
- Irion, D.N., Schaffer, A.L., Famula, T.R., Eggleston, M.L., Hughes, S.S. & Pedersen, N.C. (2003). Analysis of Genetic Variation in 28 Dog Breed Populations With 100 Microsatellite Markers. *Journal of Heredity*, vol. 94 (1), pp. 81–87
- Kim, K.-H. & Lee, K.-A. (2014). Maternal effect genes: Findings and effects on mouse embryo development. *Clinical and Experimental Reproductive Medicine*, vol. 41 (2), pp. 47–61
- Klingensmith, J. & Nusse, R. (1994). Signaling by wingless in *Drosophila*. *Developmental Biology*, vol. 166 (2), pp. 396–414
- Koch, D.A., Arnold, S., Hubler, M. & Montavon, P.M. (2003). Brachycephalic Syndrome in Dogs. p. 7
- Leroy, G. (2011). Genetic diversity, inbreeding and breeding practices in dogs: Results from pedigree analyses. *The Veterinary Journal*, vol. 189 (2), pp. 177–182 (Special Issue: Canine Genetics)
- Leroy, G., Verrier, E., Meriaux, J.C. & Rognon, X. (2009). Genetic diversity of dog breeds: within-breed diversity comparing genealogical and molecular data. *Animal Genetics*, vol. 40 (3), pp. 323–332
- Lindblad-Toh, K., Wade, C.M., Mikkelsen, T.S., Karlsson, E.K., Jaffe, D.B., Kamal, M., Clamp, M., Chang, J.L., Kulbokas Iii, E.J., Zody, M.C., Mauceli, E., Xie, X., Breen, M., Wayne, R.K., Ostrander, E.A., Ponting,

C.P., Galibert, F., Smith, D.R., deJong, P.J., Kirkness, E., Alvarez, P., Biagi, T., Brockman, W., Butler, J., Chin, C.-W., Cook, A., Cuff, J., Daly, M.J., DeCaprio, D., Gnerre, S., Grabherr, M., Kellis, M., Kleber, M., Bardeleben, C., Goodstadt, L., Heger, A., Hitte, C., Kim, L., Koepfli, K.-P., Parker, H.G., Pollinger, J.P., Searle, S.M.J., Sutter, N.B., Thomas, R., Webber, C., Broad Sequencing Platform Members, Baldwin, J., Abebe, A., Abouelleil, A., Aftuck, L., Ait-zahra, M., Aldredge, T., Allen, N., An, P., Anderson, S., Antoine, C., Arachchi, H., Aslam, A., Ayotte, L., Bachantsang, P., Barry, A., Bayul, T., Benamara, M., Berlin, A., Bessette, D., Blitshteyn, B., Bloom, T., Blye, J., Boguslavskiy, L., Bonnet, C., Boukhgalter, B., Brown, A., Cahill, P., Calixte, N., Camarata, J., Cheshatsang, Y., Chu, J., Citroen, M., Collymore, A., Cooke, P., Dawoe, T., Daza, R., Decktor, K., DeGray, S., Dhargay, N., Dooley, K., Dooley, K., Dorje, P., Dorjee, K., Dorris, L., Duffey, N., Dupes, A., Egbiremolen, O., Elong, R., Falk, J., Farina, A., Faro, S., Ferguson, D., Ferreira, P., Fisher, S., FitzGerald, M., Foley, K., Foley, C., Franke, A., Friedrich, D., Gage, D., Garber, M., Gearin, G., Giannoukos, G., Goode, T., Goyette, A., Graham, J., Grandbois, E., Gyaltzen, K., Hafez, N., Hagopian, D., Hagos, B., Hall, J., Healy, C., Hegarty, R., Honan, T., Horn, A., Houde, N., Hughes, L., Hunnicutt, L., Husby, M., Jester, B., Jones, C., Kamat, A., Kanga, B., Kells, C., Khazanovich, D., Kieu, A.C., Kisner, P., Kumar, M., Lance, K., Landers, T., Lara, M., Lee, W., Leger, J.-P., Lennon, N., Leuper, L., Levine, S., Liu, J., Liu, X., Lokyitsang, Y., Lokyitsang, T., Lui, A., Macdonald, J., Major, J., Marabella, R., Maru, K., Matthews, C., McDonough, S., Mehta, T., Meldrim, J., Melnikov, A., Meneus, L., Mihalev, A., Mihova, T., Miller, K., Mittelman, R., Mlenga, V., Mulrain, L., Munson, G., Navidi, A., Naylor, J., Nguyen, T., Nguyen, N., Nguyen, C., Nguyen, T., Nicol, R., Norbu, N., Norbu, C., Novod, N., Nyima, T., Olandt, P., O'Neill, B., O'Neill, K., Osman, S., Oyono, L., Patti, C., Perrin, D., Phunkhang, P., Pierre, F., Priest, M., Rachupka, A., Raghuraman, S., Rameau, R., Ray, V., Raymond, C., Rege, F., Rise, C., Rogers, J., Rogov, P., Sahalie, J., Settipalli, S., Sharpe, T., Shea, T., Sheehan, M., Sherpa, N., Shi, J., Shih, D., Sloan, J., Smith, C., Sparrow, T., Stalker, J., Stange-Thomann, N., Stavropoulos, S., Stone, C., Stone, S., Sykes, S., Tchuinga, P., Tenzing, P., Tesfaye, S., Thoulutsang, D., Thoulutsang, Y., Topham, K., Topping, I., Tsamla, T., Vassiliev, H., Venkataraman, V., Vo, A., Wangchuk, T., Wangdi, T., Weiland, M., Wilkinson, J., Wilson, A., Yadav, S., Yang, S., Yang, X., Young, G., Yu, Q., Zainoun, J., Zembek, L., Zimmer, A. & Lander, E.S. (2005). Genome sequence, comparative analysis and haplotype structure of the domestic dog. *Nature*, vol. 438 (7069), pp. 803–819

Liu, N.-C., Oechtering, G.U., Adams, V.J., Kalmar, L., Sargan, D.R. & Ladlow, J.F. (2017). Outcomes and prognostic factors of surgical treatments for brachycephalic obstructive airway syndrome in 3 breeds: LIU et al. *Veterinary Surgery*, vol. 46 (2), pp. 271–280

Liu, P., Lu, J., Cardoso, W.V. & Vaziri, C. (2008). The SPARC-related Factor SMOC-2 Promotes Growth Factor-induced Cyclin D1 Expression and

- DNA Synthesis via Integrin-linked Kinase. *Molecular Biology of the Cell*, vol. 19 (1), pp. 248–261
- MacDonald, B.T., Tamai, K. & He, X. (2009). Wnt/ β -catenin signaling: components, mechanisms, and diseases. *Developmental cell*, vol. 17 (1), pp. 9–26
- Maier, S., Paulsson, M. & Hartmann, U. (2008). The widely expressed extracellular matrix protein SMOC-2 promotes keratinocyte attachment and migration. *Experimental Cell Research*, vol. 314 (13), pp. 2477–2487
- Mansour, T.A., Lucot, K., Konopelski, S.E., Dickinson, P.J., Sturges, B.K., Vernau, K.L., Choi, S., Stern, J.A., Thomasy, S.M., Döring, S., Verstraete, F.J.M., Johnson, E.G., York, D., Rebhun, R.B., Ho, H.-Y.H., Brown, C.T. & Bannasch, D.L. (2018). Whole genome variant association across 100 dogs identifies a frame shift mutation in DISHEVELLED 2 which contributes to Robinow-like syndrome in Bulldogs and related screw tail dog breeds. *PLoS Genetics*, vol. 14 (12). DOI: <https://doi.org/10.1371/journal.pgen.1007850>
- Marchant, T.W., Johnson, E.J., McTeir, L., Johnson, C.I., Gow, A., Liuti, T., Kuehn, D., Svenson, K., Bermingham, M.L., Drögemüller, M., Nussbaumer, M., Davey, M.G., Argyle, D.J., Powell, R.M., Guilherme, S., Lang, J., Ter Haar, G., Leeb, T., Schwarz, T., Mellanby, R.J., Clements, D.N. & Schoenebeck, J.J. (2017). Canine Brachycephaly Is Associated with a Retrotransposon-Mediated Missplicing of SMOC2. *Current Biology*, vol. 27 (11), pp. 1573-1584.e6
- Massagué, J., Blain, S.W. & Lo, R.S. (2000). TGF β Signaling in Growth Control, Cancer, and Heritable Disorders. *Cell*, vol. 103 (2), pp. 295–309
- Mcmanus, C., A. Silva, J., S. Mariante, A., Sereno, J.R.B., Santos, S., Abreu, U., Miserani, M. & C. Mazza, M. (2002). Heritability estimates for biometric measures of the Pantaneiro horse. *Archivos de zootecnia, ISSN 0004-0592, Vol. 51, Nº 193-194, 2002,*
- Mellersh, C. (2008). Give a dog a genome. *The Veterinary Journal*, vol. 178 (1), pp. 46–52
- Melvin, V.S., Feng, W., Hernandez-Lagunas, L., Artinger, K.B. & Williams, T. (2013). A Morpholino-based screen to identify novel genes involved in craniofacial morphogenesis. *Developmental dynamics : an official publication of the American Association of Anatomists*, vol. 242 (7), pp. 817–831
- Miller, J.R. (2001). The Wnts. *Genome Biology*, vol. 3 (1), p. reviews3001.1
- Mopsorden (2015). Rasspecific Avelsstrategi för mops. Svenska Kennelklubben. Available at: <https://www.skk.se/globalassets/dokument/rasdokument/ras-mops.pdf> [2019-06-05]
- Na, J., Lykke-Andersen, K., Torres Padilla, M.E. & Zernicka-Goetz, M. (2007). Dishevelled proteins regulate cell adhesion in mouse blastocyst and serve to monitor changes in Wnt signaling. *Developmental Biology*, vol. 302 (1), pp. 40–49
- Njikam, I.N., Huault, M., Pirson, V. & Detilleux, J. (2009). The Influence of Phylogenetic Origin on the Occurrence of Brachycephalic Airway Obstruction Syndrome in a Large Retrospective Study. *The International Journal of Applied research in Veterinary Medicine*, vol. 7 (3), pp. 138–143

- Ornitz, D.M. & Itoh, N. (2015). The Fibroblast Growth Factor signaling pathway. *Wiley Interdisciplinary Reviews. Developmental Biology*, vol. 4 (3), pp. 215–266
- Ostrander, E.A. & Wayne, R.K. (2005). The canine genome. *Genome Research*, vol. 15 (12), pp. 1706–1716
- Packer, R.M.A., Hendricks, A., Tivers, M.S. & Burn, C.C. (2015). Impact of Facial Conformation on Canine Health: Brachycephalic Obstructive Airway Syndrome. *PLOS ONE*, vol. 10 (10), p. e0137496
- Parker, H.G., VonHoldt, B.M., Quignon, P., Margulies, E.H., Shao, S., Mosher, D.S., Spady, T.C., Elkahoun, A., Cargill, M., Jones, P.G., Maslen, C.L., Acland, G.M., Sutter, N.B., Kuroki, K., Bustamante, C.D., Wayne, R.K. & Ostrander, E.A. (2009). An Expressed Fgf4 Retrogene Is Associated with Breed-Defining Chondrodysplasia in Domestic Dogs. *Science (New York, N.Y.)*, vol. 325 (5943), pp. 995–998
- Pedersen, N.C., Pooch, A.S. & Liu, H. (2016). A genetic assessment of the English bulldog. *Canine Genetics and Epidemiology*, vol. 3 (1). DOI: <https://doi.org/10.1186/s40575-016-0036-y>
- Projektgruppen för engelsk bulldogg (2015). Rasspecifik avelsstrategi RAS för Engelsk bulldogg. Svenska kennelklubben. Available at: <https://www.skk.se/globalassets/dokument/rasdokument/ras-engelsk-bulldogg.pdf> [2019-06-07]
- Riecks, T.W., Birchard, S.J. & Stephens, J.A. (2007). Surgical correction of brachycephalic syndrome in dogs: 62 cases (1991–2004). *Journal of the American Veterinary Medical Association*, vol. 230 (9), pp. 1324–1328
- Rocnik, E.F., Liu, P., Sato, K., Walsh, K. & Vaziri, C. (2006). The Novel SPARC Family Member SMOC-2 Potentiates Angiogenic Growth Factor Activity. *Journal of Biological Chemistry*, vol. 281 (32), pp. 22855–22864
- Salmon Hillbertz, N.H.C., Isaksson, M., Karlsson, E.K., Hellmén, E., Pielberg, G.R., Savolainen, P., Wade, C.M., von Euler, H., Gustafson, U., Hedhammar, Å., Nilsson, M., Lindblad-Toh, K., Andersson, L. & Andersson, G. (2007). Duplication of *FGF3*, *FGF4*, *FGF19* and *ORAOV1* causes hair ridge and predisposition to dermoid sinus in Ridgeback dogs. *Nature Genetics*, vol. 39 (11), pp. 1318–1320
- Sánchez-Molano, E., Pong-Wong, R., Clements, D.N., Blott, S.C., Wiener, P. & Woolliams, J.A. (2015). Genomic prediction of traits related to canine hip dysplasia. *Frontiers in Genetics*, vol. 6. DOI: <https://doi.org/10.3389/fgene.2015.00097>
- Sargolzaei, M., Iwaisaki, H. & Colleau, J. (2006). CFC: A tool for Monitoring Genetic Diversity. *Proceedings of 8th World Congress on Genetics Applied to Livestock Production (WCGALP)*, Brazil, August 13 2006. Brazil: Belo Horizonte
- Schoenebeck, J.J., Hutchinson, S.A., Byers, A., Beale, H.C., Carrington, B., Faden, D.L., Rimbault, M., Decker, B., Kidd, J.M., Sood, R., Boyko, A.R., Iii, J.W.F., Wayne, R.K., Bustamante, C.D., Ciruna, B. & Ostrander, E.A. (2012). Variation of BMP3 Contributes to Dog Breed Skull Diversity. *PLOS Genetics*, vol. 8 (8), p. e1002849

- Schoenebeck, J.J. & Ostrander, E.A. (2013). The Genetics of Canine Skull Shape Variation. *Genetics*, vol. 193 (2), pp. 317–325
- Stockard, C., R. (1941). *The Genetic and endocrinic basis for differences in form and behaviour*. Philadelphia: The Wistar Institute of Anatomy and Biology.
- Svenska Bostonterrierklubben (2011). Rasspecifik avelsstrategi för bostonterrier. Svenska Kennelklubben. Available at: <https://www.skk.se/globalassets/dokument/rasdokument/ras-bostonterrier.pdf> [2019-06-05]
- Svenska kennelklubben (2017). *Besiktningsintyg för valpar / För kliniker*. Available at: <https://www.skk.se/sv/SKK-klinikwebb/Halsointyg/Valpbesiktningsintyg/> [2019-05-14]
- Svenska kennelklubben (2019). *Protokoll fört vid sammanträde med Svenska Kennelklubbens avelskommitté*. (1-2019). Rotebro. Available at: https://www.skk.se/globalassets/dokument/protokoll/avelskommittent/avelskommittent-1-2019.pdf?t_id=1B2M2Y8AsgTp-gAmY7PhCfg%3d%3d&t_q=brakycefali&t_tags=andquery-match%2clanguage%3asv%7clanguage%3a7D2DA0A9FC754533B091FA6886A51C0D%2csiteid%3a821afd82-44fd-4b1f-8345-260bc2345d00%7csiteid%3a84BFAF5C52A349A0BC61A9FFB6983A66%2clanguage%3asv&t_ip=192.168.20.142&t_hit.id=Skk_Skk_se_Business_Models_Media_GenericMedia/_88420198-243f-4781-ab20-ab8ae65f1bdf&t_hit.pos=5 [2019-05-14]
- Svenska kennelklubben *Registrering av operativa ingrepp / Svenska Kennelklubben*. Available at: <https://www.skk.se/sv/uppfodning/SKKs-arbete-for-sund-avel/trubbnosiga-hundar/registrering-av-operativa-ingrepp/> [2019-05-14]
- Tadjuidje, E., Cha, S.-W., Louza, M., Wylie, C. & Heasman, J. (2011). The functions of maternal Dishevelled 2 and 3 in the Early *Xenopus* embryo. *Developmental Dynamics*, vol. 240 (7), pp. 1727–1736
- Termine, J.D., Kleinman, H.K., Whitson, S.W., Conn, K.M., McGarvey, M.L. & Martin, G.R. (1981). Osteonectin, a bone-specific protein linking mineral to collagen. *Cell*, vol. 26 (1 Pt 1), pp. 99–105
- Thalmann, O., Shapiro, B., Cui, P., Schuenemann, V.J., Sawyer, S.K., Greenfield, D.L., Germonpré, M.B., Sablin, M.V., López-Giráldez, F., Domingo-Roura, X., Napierala, H., Uerpman, H.-P., Loponte, D.M., Acosta, A.A., Giemsch, L., Schmitz, R.W., Worthington, B., Buikstra, J.E., Druzhkova, A., Graphodatsky, A.S., Ovodov, N.D., Wahlberg, N., Freedman, A.H., Schweizer, R.M., Koepfli, K.-P., Leonard, J.A., Meyer, M., Krause, J., Pääbo, S., Green, R.E. & Wayne, R.K. (2013). Complete Mitochondrial Genomes of Ancient Canids Suggest a European Origin of Domestic Dogs. *Science*, vol. 342 (6160), pp. 871–874
- The Nordic Kennel Club (2018). *Breed Specific Instructions (BSI) regarding exaggerations in pedigree dogs*. (second edition) Available at: <https://www.skk.se/globalassets/dokument/utstallning/breed-special-specific-instructions--bsi-a8.pdf>
- ThermoFisher Scientific *How TaqMan Assays Work - SE*. Available at: <https://www.thermofisher.com/uk/en/home/life-science/pcr/real-time->

pcr/real-time-pcr-learning-center/real-time-pcr-basics/how-taqman-assays-work.html [2019-05-13]

- Vannahme, C., Gössling, S., Paulsson, M., Maurer, P. & Hartmann, U. (2003). Characterization of SMOC-2, a modular extracellular calcium-binding protein. *Biochemical Journal*, vol. 373 (3), pp. 805–814
- Vila, C. (1997). Multiple and Ancient Origins of the Domestic Dog. *Science*, vol. 276 (5319), pp. 1687–1689
- Wang, G.-D., Zhai, W., Yang, H.-C., Wang, L., Zhong, L., Liu, Y.-H., Fan, R.-X., Yin, T.-T., Zhu, C.-L., Poyarkov, A.D., Irwin, D.M., Hytönen, M.K., Lohi, H., Wu, C.-I., Savolainen, P. & Zhang, Y.-P. (2016). Out of southern East Asia: the natural history of domestic dogs across the world. *Cell Research*, vol. 26 (1), pp. 21–33
- Wayne, R.K. (1993). Molecular evolution of the dog family. *Trends in Genetics*, vol. 9 (6), pp. 218–224
- Xing, Y.-Y., Cheng, X.-N., Li, Y.-L., Zhang, C., Saquet, A., Liu, Y.-Y., Shao, M. & Shi, D.-L. (2018). Mutational analysis of dishevelled genes in zebrafish reveals distinct functions in embryonic patterning and gastrulation cell movements. *PLoS Genetics*, vol. 14 (8). DOI: <https://doi.org/10.1371/journal.pgen.1007551>

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Sincerely

Elin

Appendix A Laboratory Protocols

Protocol 1. PCR protocol for GAPDH

Component	Volume/reaction (μL)	Final concentration
HotStar 10X buffer	1	1X
dNTP (25mM)	0.1	250 μM
Primer F (10 μM)	0.5	0.5 μM
Primer R (10 μM)	0.5	0.5 μM
HotStarTaq	0.125	-
RNase-free water	6.775	-
DNA	1	variable
Total reaction volume	10	-

PCR protocol

Stage	Temp	Time
Hold	94°C	10min
Cycle (30 cycles)	94°C	30sec
	60°C	45sec
	72°C	1 min
Hold	72°C	10min
Hold	4 °C	∞

Protocol 2. Protocol for preparing samples for DNA extraction, using QIASymphony® DSP DNA Mini Kit

Preparing the samples:

1. Place the cytobrush in a 2 ml Sarsted Screw skirt tube.
2. Add 220 μL of ATL buffer over the brush to rinse it. Tilt the tube and swirl the brush to rinse it further.
3. Add 20 μL of Proteinase K (20mg/ml) to the tube. Tilt the tube and swirl the brush for mixing.
4. Cut the end of the brush so that it fit into the tube, screw on the lid and vortex the tube. Centrifuge the tube if to much foam.
5. Place the tube in a ThermoShaker and incubate at 56 °C and 900 rpm shake for 1 hour.
6. Centrifuge the tubes and press the brush to the tube wall to squeeze out the fluid. Remove the brush, the sample is now ready for extraction.
7. Place the sample in the QIASymphony SP automated system.

Procedure DNA extraction using DNA QIASymphony SP automated system (QIASymphony® DNA Handbook, pages 18-20).

Procedure

1. Close all drawers and the hood.
2. Switch on the QIASymphony SP and wait until the “Sample Preparation” screen appears, and the initialization procedure has finished.
3. Log on to the instrument.
4. Ensure the “Waste” drawer is loaded properly and perform an inventory scan of the “Waste” drawer, including the tip chute and liquid waste. Replace the tip disposal bag if necessary.
5. Load the required elution rack into the “Eluate” drawer.
6. Load the required reagent cartridge(s) and consumables into the “Reagents and Consumables” drawer.
7. Perform an inventory scan of the “Reagents and Consumables” drawer.
8. Place the samples into the appropriate sample carrier and load them into the “Sample” drawer.
9. For Virus Blood applications: The tube(s) containing the internal control–Buffer ATE mixture should be placed in slot A of the “Sample” drawer.
10. Using the touchscreen, enter the required information for each batch of samples to be processed.
11. Press the “Run” button to start the purification procedure. All processing steps are fully automated. At the end of the protocol run, the status of the batch changes from “RUNNING” to “COMPLETED”
12. Retrieve the elution rack containing the purified nucleic acids from the “Eluate” drawer. The DNA is ready to use or can be stored at 2–8°C, –20°C, or –80°C.

Protocol 3. PCR reaction set up for SMOC2

Table a. *Reaction setup using HotStarTaq® (Qiagen)*

Component	Volume/reaction (µL)	Final concentration
HotStar 10X buffer	1	1X
dNTP	0.1	300 µM
Primer F (10 µM)	0.3	0.3 µM
Primer RA (10 µM)	0.3	0.3 µM
Primer RD (10 µM)	0.3	0.3 µM
HotStarTaq	0.125	-
RNase-free water	6.875	-
DNA	1	variable
Total reaction volume	10	-

Table b. *Oligonucleotides used for SMOC2*

Primer	Gene	Primer sequence, 5' – 3'	GC content (%)	Tm (°C)
SMOC2 Forward	SPARC-related modular calcium binding gene	GGC AGG GGA TGG GGA AGC CT	70	60
SMOC2 Reverse (Derived)	SPARC-related modular calcium binding gene	TGC CCT AAA GTT CAG GGT CCA CT	50	57.4
SMOC2 Reverse (Ancestral)	SPARC-related modular calcium binding gene	ACT GTG TGC TTG CCC AAA CTC A	47.8	55.3

Primers were purchased desalted from TAGC Copenhagen.

Table c. 2 % Agarose gel:

Component	Small gel	Medium gel	Big gel
1 x TBE	50 µL	100 µL	200 µL
Agarose	1 gram	2 grams	4 grams
GelRed® nucleic acid gel stain	3 µL	6 µL	12 µL

Add appropriate amount of 1xTBE and agarose to a container (i.g. an Erlenmeyer Flask) with at least double the volume. Heat in the microwave until the liquid is clear. Cool the liquid to about 60°C and add the GelRed®. Pour the agarose in a tray, place combs. Cool until solid.

Protocol 4. BigDye® Direct Cycle Sequencing Kit (Applied Biosystems®, Carlsbad, USA) 02/2011 (Rev. C)

Prepare and run PCR reactions:

1. For each forward or reverse reaction, add the components to an appropriate reaction plate:

Component	Volume
Genomic DNA (4ng/ µL)	1.0 µL
M13-tailed PCR primer mix (0.8 µM each primer)	1.5 µL
BigDye® Direct PCR Master Mix	5.0 µL
Deionized water	2.5 µL
Total volume for each reaction	10.0 µL

2. Pipet up and down to mix well, seal the plate with adhesive film or caps, then spin the plate briefly.
3. Run the reactions in a thermal cycler

Stage	Temp	Time
Hold	95°C	10min
Cycle (35 cycles)	96°C	3sec
	62°C	15sec
	68°C	30sec
Hold	72°C	2min
Hold	4 °C	∞

- Optional stopping point. The amplified DNA can be stored at 4°C overnight or at -15°C or -25°C for long-term storage.

Perform cycle sequencing:

- Prepare a forward or reverse sequencing reaction mix in a tube on ice:

Component	Volume
BigDye® Direct Sequencing Master Mix	2.0 µL
BigDye® Direct M13 forward primer	1.0 µL
Or	
BigDye® Direct M13 reverse primer	
Total volume for each reaction	3.0 µL

- For each sequencing reaction, add 3 µL of the sequencing reaction mix to the appropriate well in the respective forward or reverse reaction plate.
- Seal the reaction plate with adhesive film or caps, then spin the plate briefly.
- Run the reactions in a thermal cycler:

Stage	Temp	Time
Hold	37°C	15min
Hold	80°C	2min
Hold	96°C	1min
Cycle (25 cycles)	96°C	10sec
	50°C	5sec
	60°C	75sec
Hold	4 °C	∞

- After the cycle sequencing reactions are complete, spin the plate briefly.
- Optional stopping point. The amplified DNA can be stored at 4°C overnight or at -15°C or -25°C for long-term storage.

Purify the sequencing products using the BigDye® Xterminator® Purification Kit:

1. Spin the reaction plate at 100 x g for 1 minute, then remove the seal.
2. Prepare a premix with SAM™ Solution and XTerminator® Solution in an appropriately sized tube:

Component	Volume for 1 well	Volume for 96 wells
SAM™ Solution	45 µL	4752 µL
XTerminator® Solution	10 µL	1056 µL
Total volume	55 µL	5808 µL

- a. Add the SAM™ Solution to the tube using a conventional pipette tip.
Note: Make sure there are no particulates in the SAM Solution before pipetting. If there are particulates, heat the SAM Solution to 37°C and mix to resuspend. Cool to room temperature before using.
 - b. Vortex the XTerminator® Solution bulk container at maximum speed for at least 10 seconds, until the solution is homogeneous.
 - c. Using a wide-bore pipette tip, aspirate the XTerminator® Solution. **IMPORTANT!** Avoid pipetting from the top of the liquid.
 - d. Mix the reagents until homogeneous.
3. Add 55 µL of SAM™ Solution/XTerminator® Solution premix to each well.
 4. Seal the plate using Adhesive Films, heat seal or a Septa mat.
 5. Vortex the reaction plate for 20 minutes, using the following conditions:

Vortexer	Speed
Digital Vortex-Genie® 2	1800 rpm
IKA MS3 Digital	2000 rpm
IKA Vortex 3	Setting 5
Taitec MicroMixer E-36	Maximum
Union Scientific Vertical Shaker	Setting 100
 6. In a swinging-bucket centrifuge, spin the plate at 1000 × g for 2 minutes.
 7. Optional Stopping point. If you plan to store the plate before proceeding with capillary electrophoresis, store the sample plates sealed with heat seal film or adhesive film for up to 48 hours at room temperature (20 to 25°C) or up to 10 days at 4°C or -20°C.

Appendix B, Supplementary information and Popular scientific summary in Swedish

Breed groups based on Federation Cynologique Internationale Nomenclature

Group 1: Sheepdogs and Cattle dogs

Group 2: Pinscher and Schnauser – Molossoid and Swiss Mountain and Cattle dogs

Group 3: Terriers

Group 4: Dachshunds

Group 5: Spitz and primitive types

Group 6: Scent hounds and related breeds

Group 7: Pointing Dogs

Group 8: Retrievers -Flushing Dogs – Water dogs

Group 9: Companion and Toy Dogs

Group 10: Sighthounds

Kortnosiga hundar – mer lika än olika

Hälsoproblemen hos kortskalliga och plattnosade (brakycefala) hundar, där andningssvårigheter och problem med värmereglering är vanligt, har fått stor uppmärksamhet senaste åren. Forskning har utförts för att kartlägga vilka gener som bidrar till kort nos hos hund. Genom att undersöka tre av de idag kända generna kopplade till brakycefali, visades att variationen var låg - vilket kan göra det svårt att komma till rätta med hälsoproblemen i dessa raser.

Inventering av kortnosiga hundar

För att förbättra hälsoläget för kortnosiga hundar har en inventering av bostonterrier, engelsk och fransk bulldogg samt mops gjorts i ett projekt lett av Svenska Kennelklubben. Projektet, som är en del av ett nordiskt samarbete och kommer att undersöka genetisk och exteriör variation inom varje ras. Som en del av detta projekt har den genetiska variationen inom 3 kända gener kopplade till brakycefali undersökts närmare.

Gener kopplade till brakycefali

Hittills har ett antal gener kopplade till brakycefali kartlagts. Många av dessa gener är viktiga för normal fosterutveckling, framförallt för normal utveckling av huvudet. Mutationer i dessa gener, det vill säga förändringar i cellernas genetiska material (DNA) tros ligga bakom den korta skallen hos kortnosiga hundar. Generna *SMOC2*, *BMP3* och *DVL2* undersöktes närmre i den aktuella studien.

Låg genetisk variation

Totalt ingick 102 svenska kortnosiga hundar i studien där DNA från varje hund samlades in och studerades närmare. Alla franska och engelska bulldoggar samt bostonterrier i studien visade sig bära på mutationer i alla 3 undersökta gener. Mutationen i genen *DVL2*, som även bidrar till skruvsvans, återfanns hos samtliga bulldoggar och bostonterriers men ej hos mops. Den genetiska variationen i dessa gener bedöms vara låg. Genetisk variation, det

vill säga förekomsten av genetiskt olika individer inom en population är en förutsättning för avel och naturligt urval. Genetisk variation ökar av till exempel mutationer och minskar genom avel i stängda populationer och avel mellan relaterade individer. Vid låg genetisk variation kan sjukdomar och ohälsosamma egenskaper bli vanligare. I en population med låg genetisk variation, där alla individer är genetiskt lika kan det därför vara svårt att bli av med hälsoproblem eller att selektera för nya egenskaper. Låg variation kan även göra det svårare att kunna selektera för att minska problem kopplade till utseende, som till exempel kort nos.

Vad är brakycefali?

Brakycefali hos hund kännetecknas av en kort nos, ett brett huvud och runda ögon som ofta sitter brett isär. Raser som boxer, engelsk och fransk bulldogg, bostonterrier och mops räknas ofta som brakycefala men ibland även shih-tzu, japanese chin, pekingese och griffoner. Hos kortnosiga hundar är skallen kraftigt förkortad, men rymmer samma strukturer som i en normal skalle, vilket lämnar lite plats för luft att passera. Förutom den korta nosen förekommer knipta näsborrar och andra missbildningar i luftvägarna som också kan påverka hundens andning. Nedsatt andningsförmåga påverkar även hundens förmåga att reglera sin kroppstemperatur.