Distichiasis in the Icelandic Sheepdog

Is there a correlation between the occurrence of distichiasis in the Icelandic Sheepdog and the variations in the FOXC2 and/or TWIST2 gene?

Master thesis by Rosan Bethlehem

Utrecht University, Faculty of Veterinary Medicine, Department Medicine of Companion Animals, Utrecht, the Netherlands, 2015

Supervisors:
Peter Leegwater
Micheal Boevé

Lab support:
Peter Leegwater
Manon Vos
Frank van Steenbeek

Abstract

Distichiasis is a disorder of the eyelid, which is seen in many different dog breeds. Recent numbers show a prevalence of 9 percent of distichiasis in the Dutch Icelandic Sheepdog population. Therefore an inherited background is suspected. The current method of diagnosing this disorder often gives a false negative result, which makes it unreliable for the selection of breeding dogs. In order to eliminate this disorder, a more reliable diagnosing method, like a DNA test, is desirable. Mutations in the human *FOXC2* and the *TWIST2* gene cause disorders that most commonly are presented with distichiasis: the lymphoedema-distichiasis syndrome and the Setleis syndrome respectively. Mutations in the murine *foxc2* and *twist2* gene show a similar phenotype in mice. The fact that these genes are present in dogs makes it interesting to analyze them, in order to hopefully find the genetic anomaly that causes distichiasis. DNA from sixteen Dutch Icelandic Sheepdogs (eight dogs diagnosed with distichiasis and eight negative control dogs) is collected and the *TWIST2* and the *FOXC2* genes are multiplied using PCR. The DNA is sequenced and DNA from the control dogs will be compared with DNA from the dogs diagnosed with distichiasis. Due to logistic difficulties no DNA-sequence results have been produced during the time span of this research. Since the DNA-sequencing has not been completed, more research needs to be done in order to conclude whether or not the genes are involved in distichiasis in Icelandic Sheepdogs.

Introduction

Distichiasis

Distichiasis in dogs is an inheritable condition of the eyelid where one or multiple eyelashes grow from the normally hairless eyelid margin(see fig. 1). These cilia often find their origin in the Meibomian glands and develop from undifferentiated Meibomian tissue(Gelatt 2008). This does not necessarily have to result in a problem; the cilia may curl away from the cornea and some dogs develop corneal hypoesthesia. However, more often than not ophthalmologic complications are seen(Sutkowska et al. 2012). The degree of irritation depends on the amount of cilia, the stiffness and the direction of growth of these cilia(Peter-Jones 2002). Epiphora, edema, mild conjunctivitis, blepharospasm, superficial keratitis, vascularisation and ulceration of the cornea are findings that may indicate the presence of these accessory cilia(Gelatt 2008, Peter-Jones 2002, Stades et al. 2007). The cilia can be found by a veterinarian during the examination of the eye, where the usage of a magnifying glass may be convenient. Other conditions, which should be excluded before the diagnosis "distichiasis" is made, that may present with the same kind of symptoms are conjunctivitis, ectopic cilia, keratoconjunctivitis sicca, trichiasis and entropion, although entropion may also arise secondarily to distichiasis(Gelatt 2008, Peter-Jones 2002, Stades et al. 2007). For the treatment of distichiasis several methods can be used. One of these methods is the manual removal of the cilia with forceps. The disadvantage of this method is that the cilia may grow back and the extraction of the cilia should therefore be repeated on a regular basis. Another possible method is electric epilation, where the follicle of the aberrant cilia is destroyed using diathermy via a thin wire which is placed through the hair shaft. Other surgical options are possible, including a treatment with an electro scalpel, a high frequency-hyperthermia treatment, an eyelid splitting treatment and cryosurgery(Stades et al. 2007, Chi et al. 2007, Vaughn et al. 1997).

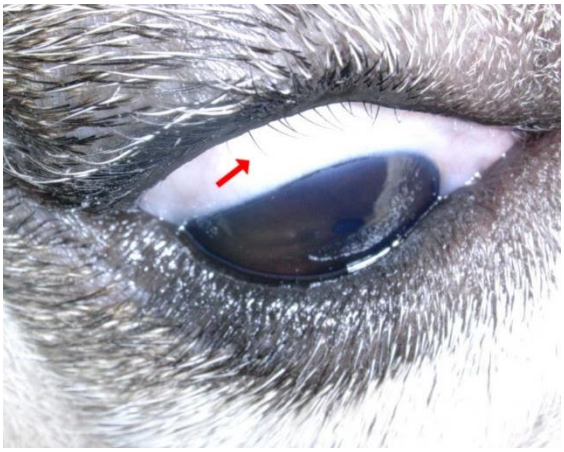

Figure 1: Red arrow shows visible distichiasis in a dogs right eye

Distichiasis and the Icelandic Sheepdog

Distichiasis is seen in many different dog breeds, research has been done on the prevalence and heritability of distichiasis in the Tibetan Terrier, the Elo and in English Cocker Spaniels(Kaufhold et al. 2007, Engelhardt et al. 2007, Kaufhold et al. 2006, Ketteritzsch et al. 2004). The Dutch Icelandic Sheepdog population also has to contend with distichiasis. At the onset of this research 270 Icelandic Sheepdogs from the approximately 1.200 registered dogs (number from Raad van Beheer 2011) in the Netherlands had been examined. 25 out of 270 of these examined Dutch Icelandic Sheepdogs were suffering from distichiasis(Vortman 2012). This represents a large sample from the Dutch population. Distichiasis is a known problem within certain breeding lines.

The problem with the existing method of diagnosing distichiasis is that it can give a false-negative result. The current breeding policy dictates that all breeding dogs need to be examined annually on the presence of distichiasis cilia. If these cilia are found, the affected dog should be excluded from

breeding programs. Unfortunately it happens that in a young dog no cilia are found during examination, while later on it may turn out that this same dog has developed distichiasis. The distichiasis cilia have a cyclic growth just like the fur, and they might have been shed on the moment of examination, which hampers the diagnosing and phenotyping of these dogs. Another problem is that the cilia may not develop until the dog is a couple of years old. This makes it possible for a dog to be falsely tested as "distichiasis-free", with consequently adverse effects on the breeding program. Because the current method of diagnosing has a low specificity, a more reliable method is desirable.

Lymphoedema-distichiasis syndrome and *FOXC2*

Lymphoedema-distichiasis syndrome (LDS) is a rare disorder in humans in which the most common clinical presentation is distichiasis followed by lymphatic and vascular abnormalities, of which lymphoedema of the lower extremities is most commonly seen. Additionally some other defects may occur, like cardiac defects, cleft palate and extradural cysts. LDS shows an autosomal dominant pattern of inheritance with variable penetrance and expression patterns(Sutkowska et al. 2012, Berry et al. 2005). In 95 percent of LDS patients a mutation on the *FOXC2* gene can be detected(Sutkowska et al. 2012). Mice that were heterozygous for a targeted disruption of *foxc2* also show distichiasis(see fig. 2)(Kriederman et al. 2003). *FOXC2* is a member of the *FOX* family of transcription factor genes. These genes generally contribute to the regulation of cellular processes like cell growth, cell differentiation, oncogenesis and metabolic homeostasis. FOX proteins generally have a Forkhead domain (FHD) and are built from three major alpha-helixes and one smaller fourth helix, plus three wing-like antiparallel beta-sheets. The third alpha-helix is also called the DNA recognition helix, it aligns itself along the major groove of DNA. The region between two beta-sheets loops around DNA and thus stabilizes protein-DNA interaction(Berry et al. 2005). *FOXC2* contributes to the development of mesenchyme cells, connective tissue, endothelium, smooth muscle cells (especially in developing blood and lymphatic vessels) and the development of the lymphatic and vascular system, in particular the maintenance of valves(Sutkowska et al. 2012). Found mutations in *FOXC2* are missense and nonsense mutations, deletions, duplications and insertions. These mutations generally cause a premature termination of the

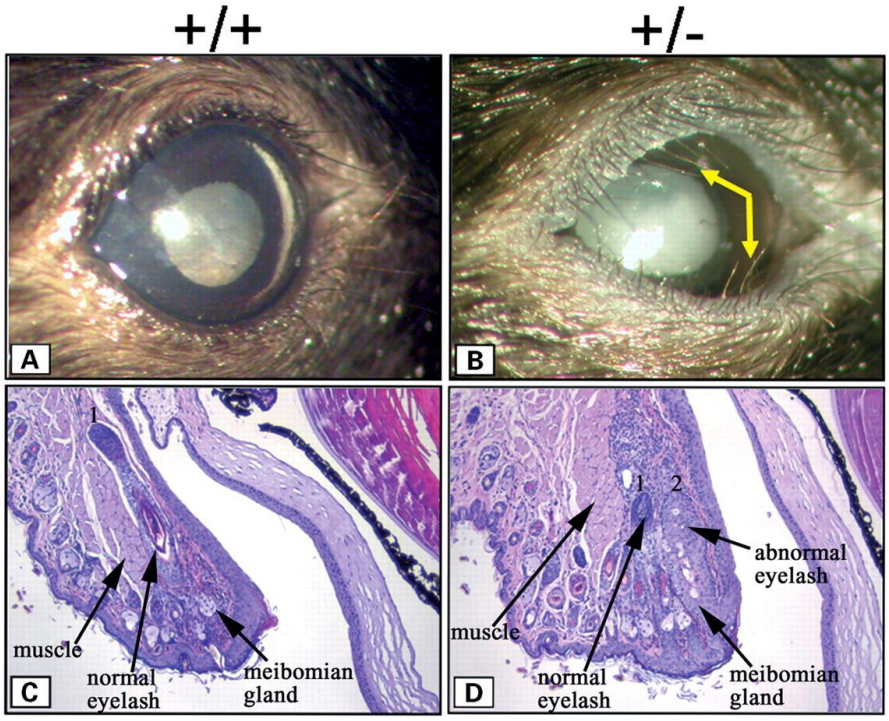

Figure 2: (A) Eyelid of a mouse with normal functioning *foxc2*; (B) Eyelid of a mouse with a heterozygous targeted disruption of *foxc2; (C)* Microscopic normal eyelid of mouse A; (D) Microscopic changes in the eyelid of a mouse with a heterozygous targeted disruption of *foxc2.* (Kriederman et al. 2003)

translation, resulting in a truncated FOXC2 protein(Berry et al. 2005, Sutkowska et al. 2012). In some cases the alpha-helical domains are eliminated and this leads to reduced *FOXC2* transcriptional activation(Sutkowska et al. 2012). An intact FOXC2 FHD is needed for development of the anterior segment of the eye. Mutations that produce an early stop codon in the FHD may therefore result in anterior segment abnormalities(Berry et al. 2005).

The Setleis syndrome and *TWIST2*

Focal facial dermal dysplasias (FFDD) are rare inherited ectodermal disorders in humans. Most commonly seen in these patients are the bitemporal or preauricular scar-like lesions, resembling forceps marks(Tukel et al. 2010, Cervantes-Barragan et al. 2011). Since the mode of inheritance of FFDD is variable (it has been described as autosomal dominant, autosomal dominant with variable expressivity and also has been described as autosomal recessive) this suggests that this syndrome is genetically heterogeneous. Subtypes of the FFDD syndrome are classified into four groups. Type I (or Brauer phenotype) includes patients with autosomal dominant inheritance of temporal lesions as the only visible feature. Type II (or Brauer-Setleis phenotype) represents patients with autosomal dominant inheritance of bitemporal lesions and Setleis like lesions, particularly distichiasis of the upper eyelids and decreased or absent lower lashes. Type III (or Setleis phenotype) includes patients with autosomal recessive inheritance of bitemporal lesions and facial or other abnormalities. Type IV represent patients with autosomal dominant or recessive inheritance of preauricular and/or cheek lesions(Cervantes-Barragan et al. 2011).

One of the facial abnormalities in Type III or the Setleis syndrome is distichiasis. Homozygosity mapping has been used to identify the gene that causes the Setleis syndrome within a Puerto Rican and an Arab family where the Setleis syndrome was seen. In order to find the exact locus of the Setleis syndrome a genome scan has been performed on five affected humans and their 26 relatives. From this mapping, homozygous *TWIST2* nonsense mutations turned out to cause the Setleis syndrome. However, sequencing *TWIST2* in five other unrelated Setleis or FFDD syndrome patients revealed that *TWIST2* was not involved in the development of the syndrome in those patients. This again suggests a genetic heterogeneity. *TWIST2* binding partners or other dermal-development transcription factors may be defective in other Setleis syndrome patients or FFDD syndromes(Tukel et al. 2010). More recent research has found that a heterozygosity for a *TWIST2* frameshift mutation that results in an truncated protein also shows an abnormal phenotype. Parents and siblings of two Mexican-Nahua Setleis patients had distichiasis of the upper eyelids and partial absence of the lower eyelashes, suggesting that heterozygosity causes a mild expression of the Setleis syndrome. A possible explanation for this might be that *TWIST2* usually inhibits facial developmental genes. Being heterozygous for this mutation may result in a slightly decreased or gradient inhibition, which results in more subtle facial lesions than homozygosity does(Cervantes-Barragan et al. 2011).

The TWIST2 protein is a member of the basic Helix-loop-helix domain (bHLH) transcription family(Tukel et al. 2010). These domains are coding for DNA binding proteins that act as transcription factors. The bHLH proteins consist of a basic area which binds DNA and two alpha-helixes which are separated by a variable loop region. The coding TWIST2 protein is called: “Twist-related protein 2”; “Twist basic helix-loop-helix transcription factor 2” or “Twist homolog 2”. A comparison of the mutated TWIST2 proteins with wild type TWIST2 proteins showed that the mutation causes a truncated and unstable protein. The mutation also causes a decreased amount of DNA binding and less transcripted proteins in comparison to the wild type(Tukel et al. 2010). The human TWIST2 and murine Twist2 proteins are 100 percent identical and *Twist2* KO mice show a similar facial phenotype as seen in Setleis syndrome patients(see fig 3) -- namely: a thin skin, sparse hair, pronounced alopecic areas between the eyes and ears, absent lower eyelashes, a narrow snout, protruding chins, short anterior-posterior head diameters and low-set dysmorphic ears. Both human *TWIST2* and murine *Twist2* may play a role in the migration and/or differentiation of neural crest cells involved in Meibomian gland and eyelash development. Cervantes-Barragan et al. concludes that the Twist-related protein 2 plays a role in mammalian facial dermal development. The shared phenotypic features in different subtypes of FFDD suggest a possible gene defect in related genes of the facial ectodermal developmental pathway(Cervantes-Barragan et al. 2011).

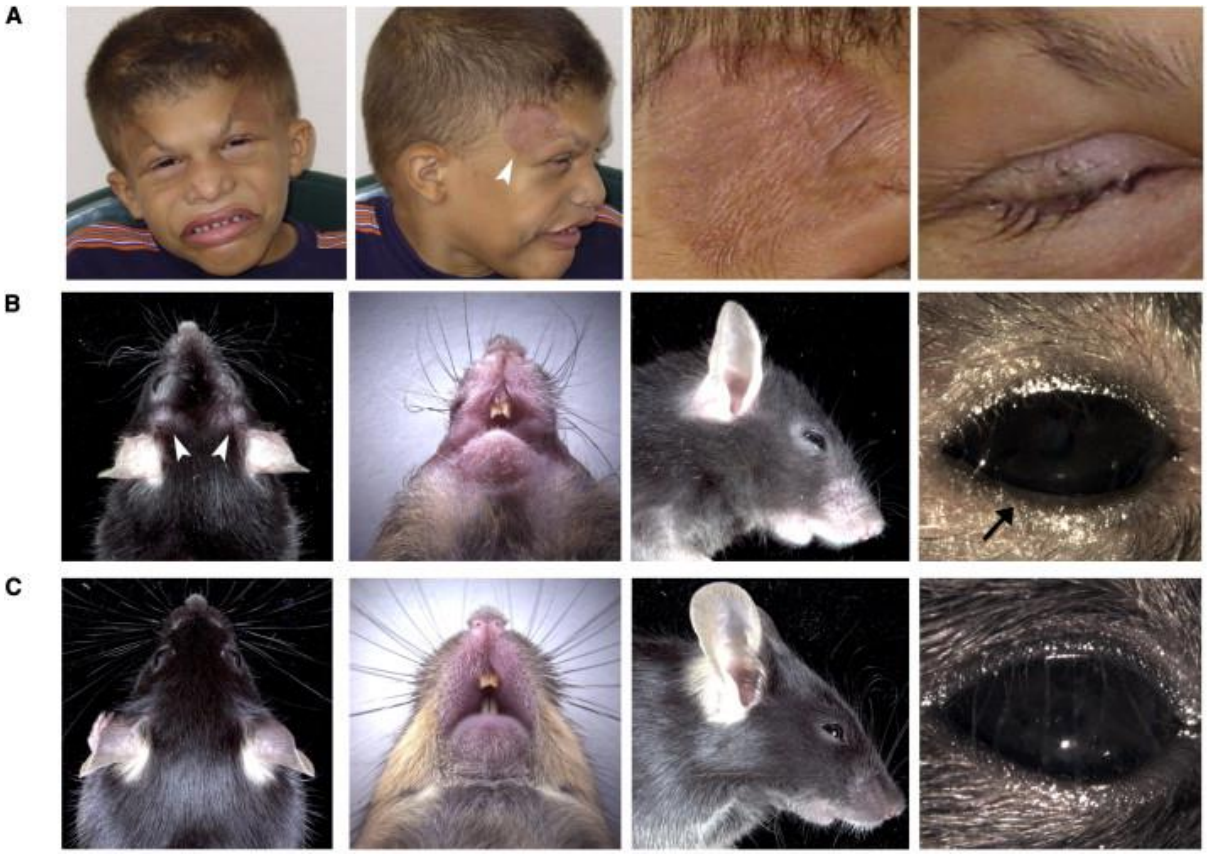

Figure 3: (A) Child with Setleis syndrome showing changes in appearance, mainly in the temporal region and in the eyelids; (B) *Twist2* knock out mouse showing changes in appearance, which match with the changes seen in humans; (C) Wildtype mouse with normal phenotype, not showing abnormalities in appearance. (Cervantes-Barragan et al. 2011)

Research aim

It would be interesting to develop the genetic defect that causes distichiasis in the Icelandic Sheepdog, in order to find a more reliable diagnostic method for this disease. Considering the linkage between mutations in human *TWIST2* and *FOXC2* and the occurrence of distichiasis,

sequencing these genes in Icelandic Sheepdog is a first step towards finding the cause of inheritable distichiasis in dogs. Therefore DNA from Icelandic Sheepdogs is isolated and sequenced to examine whether dogs with distichiasis have a mutation in the *FOXC2* or the *TWIST2* gene that is not present in the DNA of distichiasis-free dogs.

Method and material

Population

The DNA of a boxer, which was already collected by the Faculty of Veterinary Medicine of Utrecht University, was used to test which primers might be useful in order to sequence the *TWIST2* and the *FOXC2* genes. Subsequently whole blood from 16 Icelandic Sheepdogs was collected in EDTA, from which eight were diagnosed with distichiasis by a certified ECVO(European College of Veterinary Ophthalmologists) veterinarian. The other eight were also examined by a certified ECVO veterinarian, but no distichiasis was seen during examination, thus these dogs were used as a negative control. As little as possible kinship and the absence of distichiasis in these dogs and their relatives were used as a guide to select these control dogs. Informed consent and ECVO forms were collected from the owners of all these animals. The group contained both males and females from different ages and different breeders.

Material

DNA from the Icelandic Sheepdogs was isolated with the Chemagen MSM I extraction robot, according to the manufacturer's instructions. These samples were diluted 1:10 with milliQ and were used to perform PCR with the ABI GeneAmp PCR system 9700, in order to isolate and sequence the *TWIST2* and *FOXC2* genes. The primers that were used on the Icelandic Sheepdog DNA can be found in the Appendix: table 1. The *FOXC2* gene was split in four parts, of which part 1 and part 4 were amplified using a nested PCR. Phusion taq was the used polymerase in the PCR's of both *TWIST2* and *FOXC2*. Depending on the GC content of the fragment, different amounts of DMSO were used for the PCR. For the exact reaction components and programs for both the *FOXC2* and the *TWIST2* reactions, see Appendix: table 2-15. Exo1 and a BigDye Terminator Sequencing Kit have been used before the PCR products were sequenced.

Research design

The different primers are used on the DNA of the Icelandic Sheepdogs to amplify the *TWIST2* and *FOXC2* gene (see: fig. 4-5 & Appendix: table 1). The reactions and reaction programs that are used in the ABI GeneAmp PCR system 9700 can be found in the appendix (see Appendix; table 2- 15). After PCR, the samples are loaded for gel electrophoresis. If the gel electrophoresis shows a desirable result, the samples are treated with Exo1. Samples are prepared for sequencing reactions, using BigDye Terminator sequencing kit to optimize this reaction. After sequencing cycles, the products are purified by using ABI's ethanol precipitation method in 1,5 ml vials, following ABI's protocol (see Appendix). After ethanol precipitation product is transferred to 96-well plates and sequenced and analyzed on the ABI prism 3130xl Genetic Analyzer with 3130xl series data collection 4 AB, using the manufacturer's instructions.

Results

Primers & PCR *TWIST2*

The *TWIST2* gene consists of 51065 base pairs, from which 184 – 666 are coding. The gene map and the span of the amplified sequence, by using a single primer-pair (see Appendix: table 1) are shown in fig. 4. As can be seen, only one pair of primers was needed to amplify the coding part of *TWIST2.* PCR results from *TWIST2* using Bio rad Gel doc 2000 and Quantity One 4.3.0 are also shown in this figure. The PCR results show single bands.

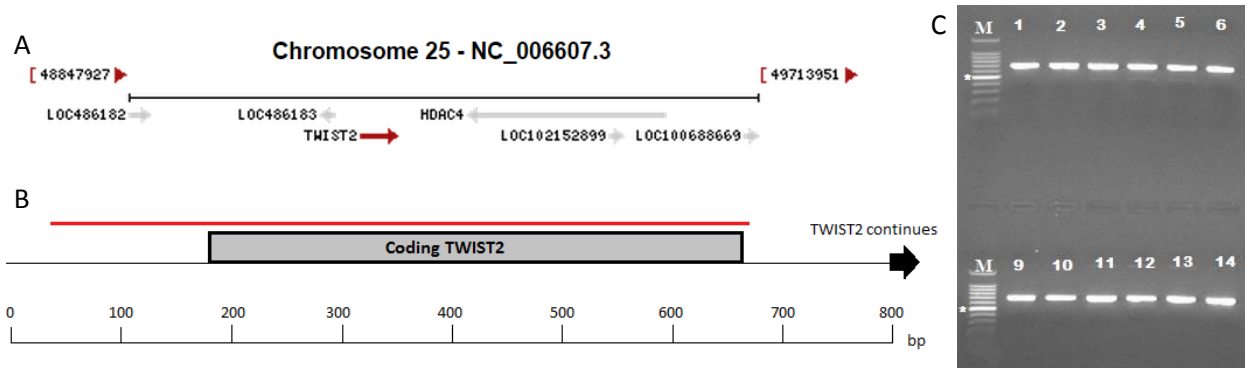

Figure 4: (A) The location of *TWIST2* on chromosome 25 is shown; (B) The coding part of *TWIST2* is shown, which is a small fragment of the entire *TWIST2* gene. The red line shows the sequence of *TWIST2* that is amplified using a single primer-pair; (C) Shows the PCR results, using this primer-pair. Each number represents the DNA of one of the Icelandic Sheepdogs used in this research, M is the 100 bp molecular weight ladder, * stands for 500 bp.

Primers & PCR *FOXC2*

Compared to the *TWIST2* gene, the *FOXC2* gene is relatively small. However, the coding part of *FOXC2* is rather large compared to *TWIST2.* Canine *FOXC2* consists of 4182 base pairs from which 70 – 1587 are coding. The gene map and the used primers to help amplifying the coding part of *FOXC2* (see Appendix: table 1) are shown in fig. 5. Considering the length of the gene, 4 primer-pairs were needed in order to amplify the sequence. The first and last primer-pairs did not show one single band after PCR, but apart from the expected band also showed multiple smaller bands. Thus was decided to use a nested PCR on these parts. The result of the nested PCR of part 1 and 4, and the PCR of part 2 and 3 using Bio rad Gel doc 2000 and Quantity One 4.3.0 gave clear single bands, which are shown in fig. 6.

Sequencing

Unfortunately, some technical difficulties occurred. Therefore, despite of the good PCR results of some fragments, it was not possible to obtain any sequence results during the time span of this research.

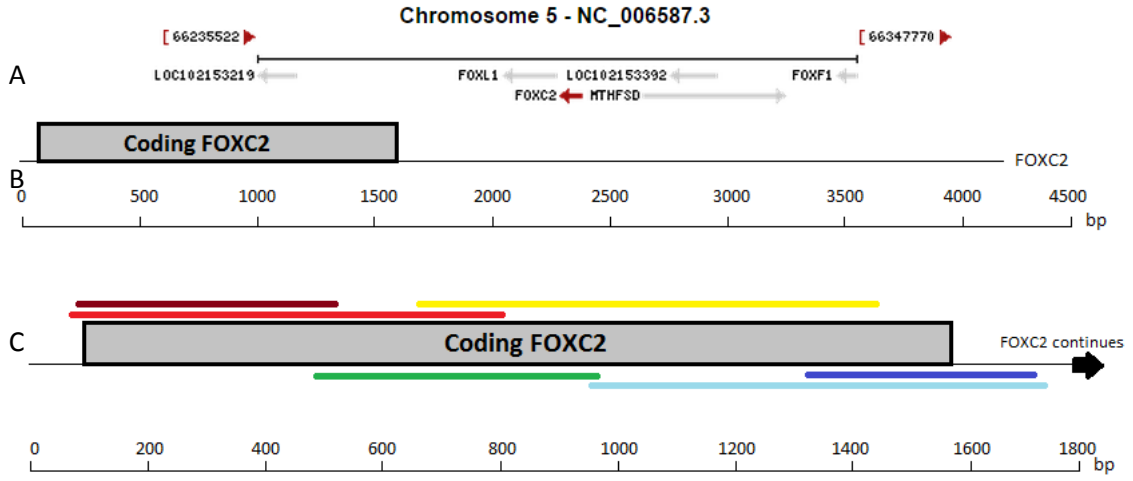

Figure 5: (A) The location of *FOXC2* on chromosome 5 is shown; (B) The coding part of *FOXC2* is shown with respect to the entire gene. (C) The extent of the used primer-pairs is shown with respect to the coding part of *FOXC2*; (D) Legend with used primer-pairs. F1N1 & R1N1 are the first nested primers of part 1; F1N2 & R1N2 are the second nested primers of part 1; F2 & R2 are the primers of part 2; F3 & R3 are the primers of part 3; F4N1 & R4N1 are the first nested primers of part 4; F4N2 & R4N2 are the second nested primers of part 4.

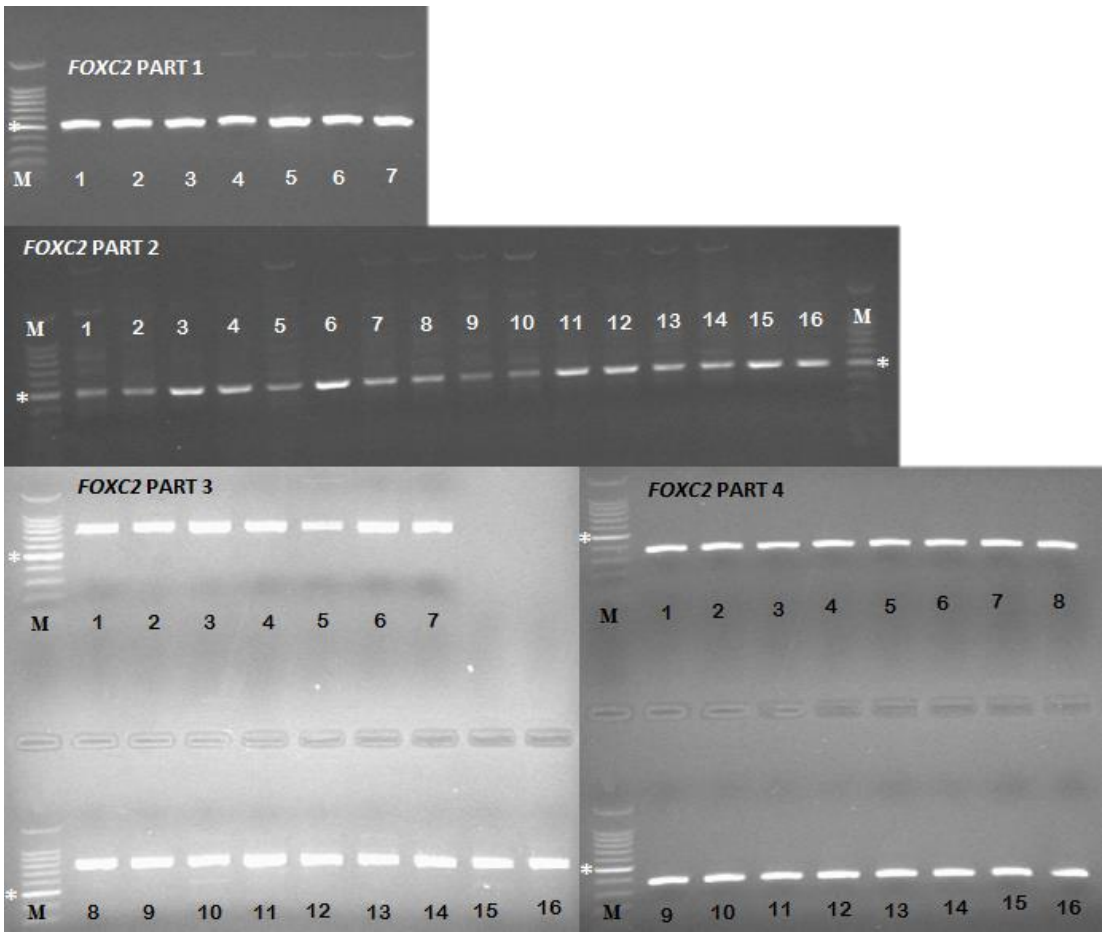

Figure 6: Shows the PCR results of *FOXC2*, using the 4 primer-pairs. Part 1 and part 4 both show the result of the second nested PCR. Each number represents the DNA of one of the Icelandic Sheepdogs used in this research, M is the 100 bp molecular weight ladder, * stands for 500 bp.

Discussion

Due to a lack of sequence results, it remains uncertain whether mutations in *FOXC2* or *TWIST2* play a role in the development of distichiasis in Icelandic Sheepdogs. In order to obtain useable results, further research is necessary. The PCR products should be sequenced and the sequenced DNA from the control dogs should be compared to the sequenced DNA from the distichiasis dogs. If this does not lead to any new insights, a whole genome scan may be considered as the next step towards finding the genetic cause of distichiasis in the Icelandic Sheepdog.

The mode of inheritance of distichiasis in the Icelandic Sheepdog remains unclear. Purely based on the pedigree of used dogs(see Appendix: fig.7), it is less likely that distichiasis is an autosomal dominant treat. After all, the pedigree shows that a distichiasis positive pup originated from a distichiasis negative male and female. This makes it more likely that it concerns an autosomal recessive or a polygenic treat. However, as stated in the introduction, it does occur that distichiasis negative tested dogs develop distichiasis later in life. Therefore, it is uncertain whether the distichiasis negative dogs in the pedigree will remain negative. This makes it difficult to say anything about the mode of inheritance, based on the pedigree. Studies on other dog breeds show different results. Research showed that distichiasis in the Elo dog is not purely caused by non-genetic factors. However, the exact mode of inheritance remains uncertain. Recessive, dominant, major gene and polygenic models all remained likely hypotheses of inheritance(Kaufhold et al. 2007). Heritability of distichiasis in the Elo is estimated to be 0,43(Kaufhold et al. 2006). Heritability in the English Cocker Spaniel is estimated at about 0.61-0.62(Engelhardt et al. 2007). In the Tibetan Terrier the heritability of distichiasis is very different from previously named breeds, research by Ketteritzsch et al. in 2004 estimated the heritability at about 0,04. This research included 849 German Tibetan Terriers from 596 litters. Apparently, the heritability of distichiasis in different breeds is variable. In order to say anything about the heritability and the mode on inheritance of distichiasis in the Icelandic Sheepdog, further breed-specific research needs to be done. Based on previous stated heritabilities, the only thing that can be concluded is that distichiasis is a heritable eye disease.

Current breeding policy dictates that all breeding dogs should be examined annually by a ECVO licensed veterinarian on the presence of distichiasis. Dogs diagnosed with distichiasis by a ECVO licensed veterinarian are excluded from all breeding purposes. This policy restricts an explosive increase of distichiasis, but is not sufficient in the complete elimination of distichiasis in the current population. The pedigree of the used distichiasis positive dogs (see Appendix; fig. 7) is an example of this. In an attempt to eliminate the prevalence of distichiasis in the current population other measures need to be taken. The Dutch association of the Icelandic Sheepdog mentioned the following options: Excluding all offspring and littermates from distichiasis-positive dogs from breeding purposes. These measures call for a strict administration of all distichiasis-positive dogs and annual evaluation of new cases. Feedback to owners of littermates or offspring of new diagnosed distichiasis dogs is desirable, if these owners are interested in breeding. The disadvantage of these measures is that the current gene pool will be narrowed. Other inheritable diseases also need to be taken account of. Considering the small Dutch Icelandic Sheepdog population the usage of international breeding lines should be encouraged, if the health of the foreign breeding dogs and their pedigrees is also strictly examined.

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Appendix

Pedigree distichiasis positive Icelandic Sheepdogs

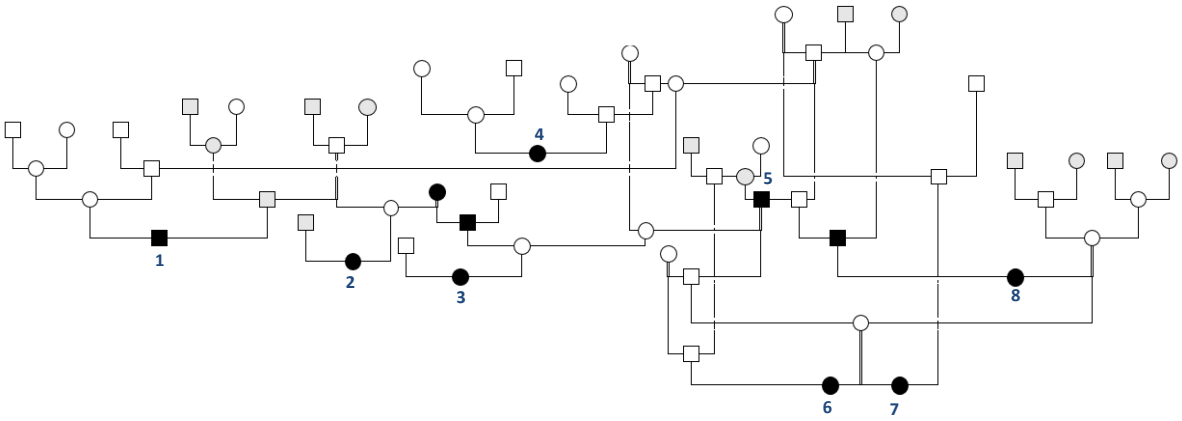

Figure 5: Pedigree of the distichiasis positive Icelandic Sheepdogs which were used in this research (numbered from 1-8). Black circles/squares: distichiasis examined positive dogs. White circles/squares: distichiasis examined negative dogs. Grey circles/squares: unknown whether these dogs are distichiasis positive or negative.

Used primers for *TWIST2* and *FOXC2*

Gene		Primer
TWIST2		F: GGAGACCTCGGTTTTGCAC R: CGCGCAGCAGCACCTACCCT
FOXC2	Part 1 - Nested 1	F: CCGGCTCCCTTCCTCTC R: CTCGCTCTTGATCACCACCT
	Part 1 - Nested 2	F: CTCCCTTCCTCTCGCTCCCT R: GGCACCTTGACGAAGCACTC
	Part 2	F: AGAACAGCATCCGCCACAAC R: GAGGTTCGCAGCGTCATGAT
	Part 3	F: GGCGCTTCAAGAAAAAGGAC R: GTCCCCGCTGTGGTTGAGAT
	Part 4 - Nested 1	F: CGGCTTCAGCGTGGAGAACA R: TTGCGTCTCTGCAGCCCGAA
	Part 4 - Nested 2	F: CACCACCAGCACCACGGCCA R: CCGAACGGTCTGAGAGGTC

Table 1: Used primers for *TWIST2* and *FOXC2*

PCR reaction components and reaction programs

TWIST2

DMSO	2,0 µL
dNTP's	0,5 µL
DNA	1,0 µL
BufferGC	5,0 µL
Primer F	1,25 µL
Primer R	1,25 µL
Phusion taq	0,25 µL
MilliQ	13,75 µL

Table 2: PCR reaction components (*TWIST2*)

98,0 ˚C	30"	
98,0 ˚C	10"	repeat 35x
68,0 ˚C	30"	
72,0 ˚C	40"	
72,0 ˚C	5'	
4,0 ˚C (optional)	∞	

Table 3: PCR reaction program (*TWIST2*)

FOXC2, part 1, nested 1

DMSO	2,5 µL
dNTP's	0,5 µL
DNA	1,0 µL
BufferGC	5,0 µL
Primer F	1,25 µL
Primer R	1,25 µL
Phusion taq	0,25 µL
MilliQ	13,25 µL

Table 4: PCR reaction components (*FOXC2*, part 1, nested 1)

98,0 ˚C	30"	
98,0 ˚C	10"	repeat 35x
65,0 ˚C	30"	
72,0 ˚C	40"	
72,0 ˚C	5'	
4,0 ˚C (optional)	∞	

Table 5: PCR reaction program (*FOXC2*, part 1, nested 1)

FOXC2, part 1, nested 2

DMSO	2,5 µL
dNTP's	0,5 µL
DNA*	1,0 µL
BufferGC	5,0 µL
Primer F	1,25 µL
Primer R	1,25 µL
Phusion taq	0,25 µL
MilliQ	13,25 µL

Table 6: PCR reaction components (*FOXC2*, part 1, nested 2); *DNA is previous PCR product 1:10

98,0 ˚C	30"	
98,0 ˚C	10"	repeat 20x
65,0 ˚C	30"	
72,0 ˚C	40"	
72,0 ˚C	5'	
4,0 ˚C (optional)	∞	

Table 7: PCR reaction program (*FOXC2*, part 1, nested 2)

FOXC2, part 2

DMSO	1,25 µL
dNTP's	0,5 µL
DNA	1,0 µL
BufferGC	5,0 µL
Primer F	1,25 µL
Primer R	1,25 µL
Phusion taq	0,25 µL
MilliQ	14,5 µL

Table 8: PCR reaction components (*FOXC2*, part 2)

98,0 ˚C	30"	
98,0 ˚C	10"	repeat 35x
65,0 ˚C	30"	
72,0 ˚C	40"	
72,0 ˚C	5'	
4,0 ˚C (optional)	∞	

Table 9: PCR reaction program (*FOXC2*, part 2)

FOXC2, part 3

DMSO	1,75 µL
dNTP's	0,5 µL
DNA	1,0 µL
BufferGC	5,0 µL
Primer F	1,25 µL
Primer R	1,25 µL
Phusion taq	0,25 µL
MilliQ	14,0 µL

Table 10: PCR reaction components (*FOXC2*, part 3)

98,0 ˚C	30"	
98,0 ˚C	10"	repeat 35x
65,0 ˚C	30"	
72,0 ˚C	40"	
72,0 ˚C	5'	
4,0 ˚C (optional)	∞	

Table 11: PCR reaction program (*FOXC2*, part 3)

FOXC2, part 4, nested 1

DMSO	2,5 µL
dNTP's	0,5 µL
DNA	1,0 µL
BufferGC	5,0 µL
Primer F	1,25 µL
Primer R	1,25 µL
Phusion taq	0,25 µL
MilliQ	13,25 µL

Table 12: PCR reaction components (*FOXC2*, part 4, nested 1)

98,0 ˚C	30"	
98,0 ˚C	10"	repeat 20x
65,0 ˚C	30"	
72,0 ˚C	40"	
72,0 ˚C	5'	
4,0 ˚C (optional)	∞	

Table 13: PCR reaction program (*FOXC2*, part 4, nested 1)

FOXC2, part 4, nested 2

DMSO	1,0 µL
dNTP's	0,5 µL
DNA*	1,0 µL
BufferGC	5,0 µL
Primer F	1,25 µL
Primer R	1,25 µL
Phusion taq	0,25 µL
MilliQ	14,75 µL

Table 14: PCR reaction components (*FOXC2*, part 4, nested 2); *DNA is previous PCR product 1:10

98,0 ˚C	30"	
98,0 ˚C	10"	repeat 35x
65,0 ˚C	30"	
72,0 ˚C	40"	
72,0 ˚C	5'	
4,0 ˚C (optional)	∞	

Table 15: PCR reaction program (*FOXC2*, part 4, nested 2)

Ethanol/EDTA Precipitation Method for BigDye Terminator protocol

For each reaction the following protocol applies:

- Add 20 µL reaction volume to an empty 1,5 mL vial;
- Add 5 µL EDTA 125mM;
- Add 70 µL ethanol 96%;
- Mix on vortex;
- Place vials in centrifuge and centrifuge cold on 13.000 rpm for 10 minutes;
- Gently discard supernatant;
- Add 200 µL of 70% ethanol;
- Place vials in centrifuge and centrifuge cold on 13.000 rpm for 5 minutes;
- Gently discard supernatant;
- Leave the sediment to air dry, on a water bath of 60˚C;
- Dissolve product in 200 µL MilliQ in order to use for sequencing.