Swedish University of Agricultural Sciences

Department of Animal Breeding and Genetics

Fine-Mapping of Equine Multiple Congenital Ocular Anomalies on horse chromosome 6

by

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Supervisors:

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Examensarbete 306

2009

Examensarbete ingår som en obligatorisk del i utbildningen och syftar till att under handledning ge de studerande träning i att självständigt och på ett vetenskapligt sätt lösa en uppgift. Föreliggande uppsats är således ett elevarbete och dess innehåll, resultat och slutsatser bör bedömas mot denna bakgrund. Examensarbete på D-nivå i ämnet husdjursgenetik, 20 p (30 ECTS).

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Contents

Abstract

Multiple Congenital Ocular Anomalities (MCOA) is a genetic disease that affects primarily Silver coloured horses of breeds such as the Rocky and Kentucky mountain horses. In these breeds the Silver dapple colour is very popular leading to an increase of affected horses. The major feature of the disease is ocular cysts of variable size. Large cysts also lead to a variety of secondary syndromes, for example retinal detachment. In previous studies the locus for *MCOA* has been mapped to an interval of 420 kb on equine chromosome six. Within this interval there are a number of genes including the *PMEL17* gene that carry the mutation that is believed to cause the Silver dapple coat color. This study has focused on shortening the interval by fine-mapping using Single Nucleotide Polymorphism (SNP) markers. This was done by recovering polymorphic markers from the EcuCab 2.0 publication of equine SNPs. The genotyping was carried out using preordered primers and probes in the ABI 7900HT fast real-time PCR system. When evaluating the results two different disease haplotypes were detected, one longer and one shorter. These recombinant chromosomes helped to decrease the interval by 37 % resulting in an interval of 265 kb. This reduced the number of genes leaving sixteen to be investigated, including the *PMEL17*. The resulting dataset concurred to the hypothesis of a codominant inheritance pattern. A gene within the interval called *SMARCC2* was chosen for sequencing due to its known importance in ocular development. The sequencing exposed a polymorphism in the intron between exon nineteen and twenty. Following studies include extended investigation of the *SMARCC2* polymorphism, sequencing of other candidate genes and functional studies of the most interesting mutations.

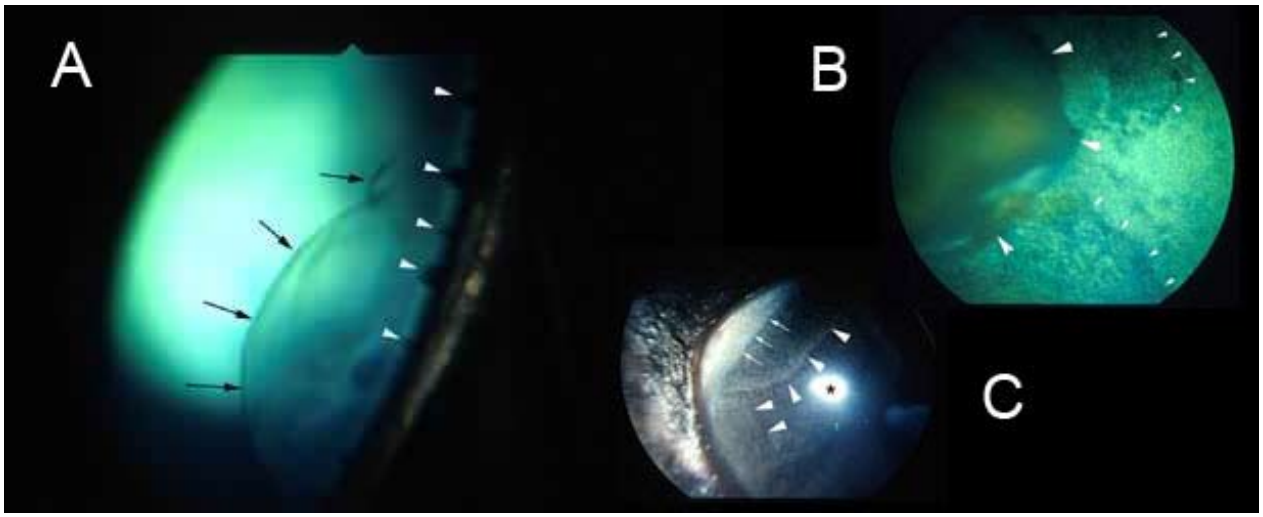
Summary in Swedish

Kartläggning av MCOA på hästens kromosom 6

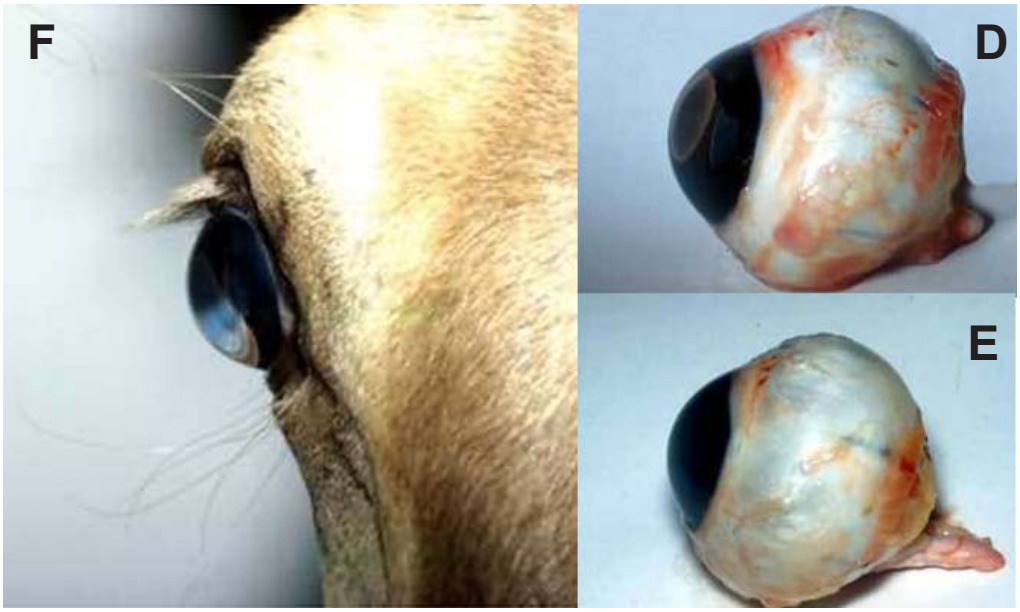
Flera Kongenitala Okulära avvikelser (MCOA) är en sjukdom som drabbar främst silverfärgade hästar av raser som Rocky och Kentucky Mountain hästar. I dessa raser är silverfärgen mycket populär vilket har lett till en ökad frekvens av drabbade hästar inom rasen. Det viktigaste sjukdomssymptomet är okulära cystor av varierande storlek. Stora cystor leder även till en rad sekundära symptom exempelvis näthinneavlossning. I tidigare studier har ett lokus för *MCOA* kartlagts till ett intervall på 420 kb på hästkromosom sex. Inom detta intervall finns det ett antal gener inklusive *PMEL17* vilken bär den orsakande mutationen för pälsfärgen silver. Denna studie har fokuserat på att korta ned kromosomintervallet med Single Nucleotide Polymorphism (SNP) markörer. Detta gjordes genom att använda polymorfa SNP markörer från EcuCab 2,0. Genotypningen gjordes med hjälp av förbeställda primers och prober i ABI 7900HT snabb realtids-PCR-system. Vid bedömningen av resultatet upptäcktes två olika sjukdomshaplotyper, en längre och en kortare. Dessa rekombinanta kromosomer har bidragit till att minska intervallet med 37 % vilket resulterar i ett intervall på 265 kb. Detta har minskat antalet gener som skall undersökas till sexton, inklusive *PMEL17*. Resultaten överensstämmer med hypotesen om ett codominant nedärvningsmönster. En gen inom intervallet, *SMARCC2*, valdes inom detta projekt för sekvensering på grund av dess inblandning i ögats utveckling. En polymorfism upptäcktes i intronet mellan exon nitton och tjugo. Fortsatta studier för att hitta den orsakande mutationen omfattar utökad undersökning av *SMARCC2* polymorfismen, sekvensering av andra kandidatgener samt funktionsstudier av de mest intressanta mutationerna.

Introduction

Ramsey *et. al.* first described congenital ocular abnormalities of the Rocky mountain horse in 1999. The most predominant features of equine Multiple Congenital Ocular Abnormalities (MCOA) are cysts of variable sizes (2-20mm) originating from the posterior iris, ciliary body and peripheral retina. These cysts are found in most horses affected by MCOA and are usually bilateral. The presences of large cysts in the eye cause a number of secondary symptoms. Another feature is dark pigmented curvilinear streaks of retinal-pigmented epithelium that cross the peripheral tapetal fundus from the peripheral retina to the optic papilla. These streaks are the remnants of previous retinal dysplasia. Veniform folds characterize retinal dysplasia of the temporal part of the peripheral retina. Total detachment of the retina was found in horses that suffered from cysts in the ciliar body. Here the retina detaches in a curvilinial streak from the ciliar body stretching toward the optic papilla. Horses suffering from some or all of these symptoms often have additional ocular abnormalities involving the angels of the eye, iris abnormalities or lenticular cataracts. All these abnormalities also lead to enlarged optical diameter and an irregular optical shape, cornea globusa, which is easily observable due to the protrusion of the eyes. Horses affected by this syndrome seem to have less impaired vision than would be expected. This could be explained in part by the fact that the intraocular pressure is within the normal range. Horses with small cysts have normal vision.



Picture 1, A, Right eye of an MCOA affected Rocky Mountain horse. Two large ciliar cysts are visible under the black arrows, the white arrows point to the elongated ciliary body. B, Retinal detachment (large arrows) is one of the symptoms of MCOA. Hyperpigmantation lines mark the borders of previous retinal detachments. C, The iridocorneal angles change due to the ocular cysts.



Picture 2, D, An eye from a MCOA affected Rocky Mountain horse. Note the size difference and the irregular form compared to a nonaffected eye. E, An eye from a non affected, age correlated Rocky Mountain horse. F, The protrution of an eye with Cornea Globosa is easily visible . © Ramsay DT.

A B

Picture 2. This is an attempt to visualize the effect that the cysts may have on the eye sight of horses affected by MCOA (B) compared to non-affected horses (A). © Ramsey DT.

In the original study of MCOA the conclusion was that the phenotype is heritable since most cases came from the same breed lineages (Ramsey *et. al.* 1999). Ewart *et. al.* 2000 studied the inheritance pattern within the Rocky mountain breed using pedigree data including 516 affected and non-affected horses. This dataset best fitted the codominance model where both homozygous and heterozygous are affected although the level of severity of the symptoms

differ. A problem occurring in the study is that heterozygous horses have very small cysts, which could lead to false negatives, as very small cysts could be difficult to detect. Recently however, another study was published with an alternative hypothesis regarding the inheritance pattern (Grahn, B *et. al.* 2008). According to this dataset, which includes 134 horses of the Rocky and the Kentucky Mountain breed, the inheritance of MCOA is autosomal dominant with incomplete penetrance. The differences of these two hypotheses can be explained in part by a slight difference in the phenotyping of the cases in the two studies and the fact that different controls were used. The latest study in the area also point to a codominant inheritance pattern (Andersson, L *et. al.* 2008). All studies do agree on the fact that Silver colored horses show the MCOA phenotype more often than would be expected by chance.

The Silver dapple coat color is caused by dilution of eumelanin. This is most clearly visible in the long hairs, mane and tail, which are diluted into a gray or flaxen color while the body hair remain darker. The silver dapple coat color is associated to a missense mutation in exon 11 in the *PMEL 17* gene (Brunberg *et. al.* 2006). *PMEL 17* mutations have been detected in many species causing different phenotypes of coat color dilutions. It is also a candidate gene for the MCOA mutation, due to the physical connection to the locus for *MCOA*, and that *PMEL17* mutations disturbance of melanin pathways cause developmental ocular anomalies in other species (Schontaler *et. al.* 2005). The reason why *MCOA* was first found in the Rocky and the Kentucky mountain horses (RH and KH) is that these breeds were recently created by using a few founding stallions. This increased the level of homozygosity. Furthermore, the extended breeding for desired traits will also increase, unevenly, the level of an undesired trait closely connected to the desired trait. In the case of RH and KH the desired trait is the Silver dapple coat color. MCOA have been detected in Silver dapple colored horses in other breeds as the Belgian draft horse, the Morgan horse, the Shetland pony and the American Miniature horse (Ramsay D, *et. al.* 1999). Including these breeds into the study may help to diminish the interval for the location of the causative mutation.

Recently the phenotype has also been detected in the Icelandic horse (Ekesten B, *et. al.* 2009). This study was performed by close opthalmologic examination of eleven Icelandic horses including both homo- and heterozygotes for the *Silver* mutation as well as non-carriers. Of the horses that carried the mutation three out of six were phenotyped as *MCOA*-affected while no cysts could be found in the eyes of the non-carriers.

A

B

C

D

Picture 3. Silver Dapple colored horses affected with MCOA from different breeds. A - Rocky mountain horse, B - American Miniature horse, C - Belgian Ardenne and D - Shetland Pony. © Ramsey DT.

Picture 4, A Silver dapple colored Icelandic stallion.

This study will add information to achieve a better understanding of the genetic components in the development of the ocular system. There are a number of ocular disorders in humans that are known to be inheritable. The precise genetic components that contribute to these diseases are still largely unknown. There is no exact human form MCOA but there are a number of disorders where the symptoms show similarities to the symptoms displayed by MCOA affected horses. One example is Anterior Segment Dysgenesis (ASD) a collection of developmental irregularities in the anterior segment of the eye (Sowden J, 2007). These symptoms resemble those portrayed by horses with MCOA. This study intended to shorten the chromosome interval for MCOA and examine the possible candidate genes within the interval. This was done by fine-mapping using single nucleotide polymorphisms (SNP). The SNPs were chosen from the list of SNPs published during the development of the equine SNP chip that is being evaluated at present (Mickelson J. *et. al.* 2009). The SNPs were detected by comparing the nucleotides at each position in the individual that was used for the genome sequencing. Additional SNPs were found in the genome by comparison to a few horses of seven different breeds selected to represent global diversity. In this way approximately 54 000 SNPs could be detected. I intend to use ten of these SNPs evenly distributed in our interval of 420 kb. This interval is found at the end of the q-arm of equine chromosome 6. It is located in an area that is gene dense and in our interval alone; there are 28 genes, one of them being the *PMEL17* gene. The intention of this project is to narrow down the interval and evaluate the genes in the new interval. To do this both MCOA-affected horsed and healthy controls will be genotyped. Since we do not have a large number of horses with known MCOA status I will add horses that can be suspected to carry the mutation, namely Silver colored horses from breeds known to carry the disease, as well as breeds that express the Silver dapple phenotype but have not yet been investigated for MCOA and non-Silver colored offspring from these Silver colored horses. The latter samples will be used in the hope of finding recombinants that can drastically reduce the interval where the causative mutation can be found. The method I have chosen is high throughput real time PCR and allelic discrimination with the ABI 7900 HT system (Bookout AL. *et. al.* 2006).

Materials and methods

Horse material

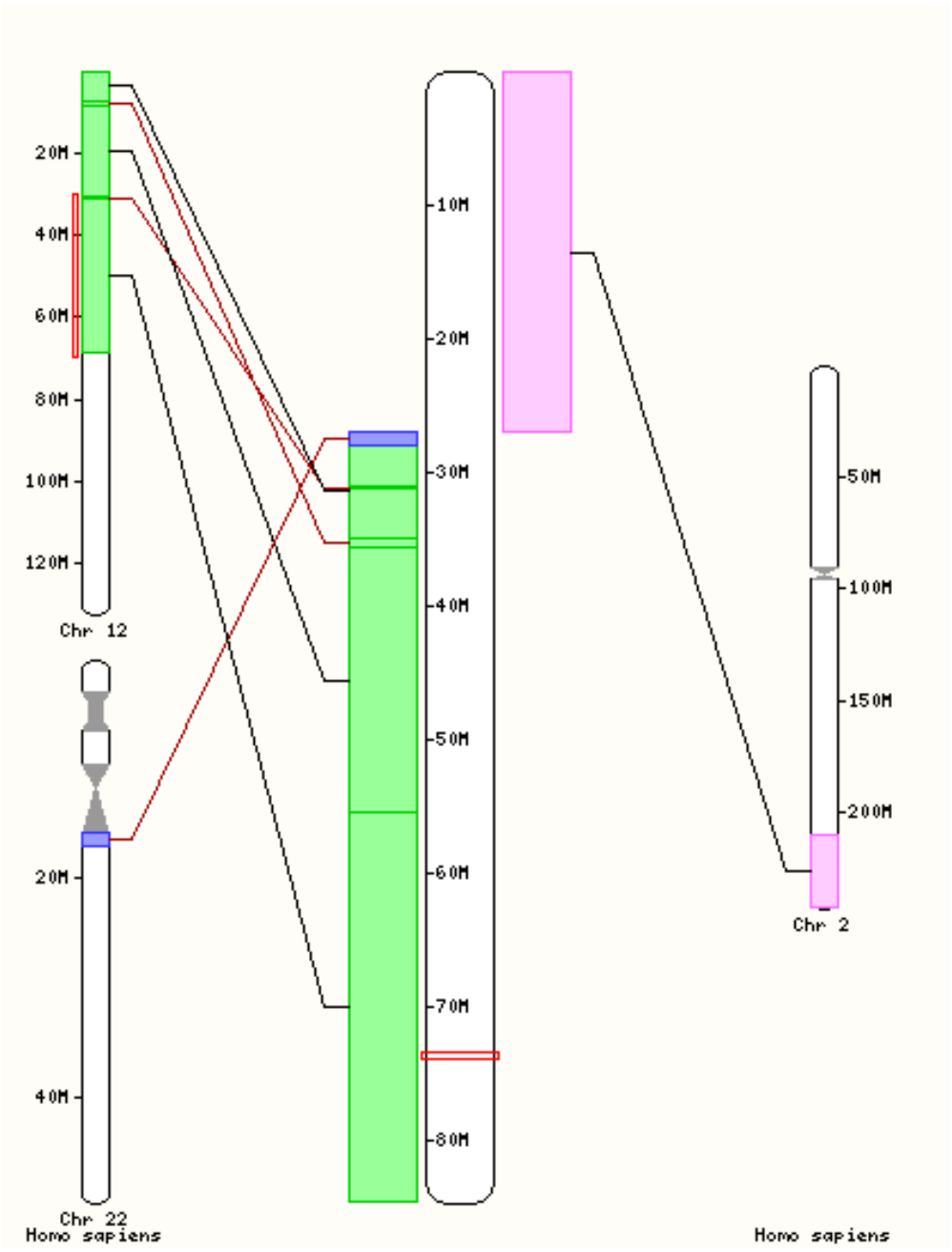
The material was chosen with the purpose to decrease the length of the interval as much as possible. Genomic DNA prepared from horses phenotyped for MCOA were reused from previous studies. These were horses with full MCOA, with cysts only and unaffected horses. The horses chosen had been found to be recombinant in some part of the interval in the previous study. Horses from the previous study that had for some reason not worked perfectly were also added to the material. One of the ideas with this project is that the causative mutation for MCOA is very close to or the same as the missense mutation in *PMEL17* that causes the dilution of eumelanin that gives the Silver dapple coat color. For this reason previously non-genotyped Silver colored horses from both breeds that are known to carry the syndrome and breeds that are believed to carry the disease were added to the material. In order to find a recombinant sample that could help decrease the interval, more drastically, non-Silver colored offspring from Silver colored horses were genotyped. In the case of a possibly interesting recombinant sample this horse will be phenotyped. In Table 1 is an overview of the material.

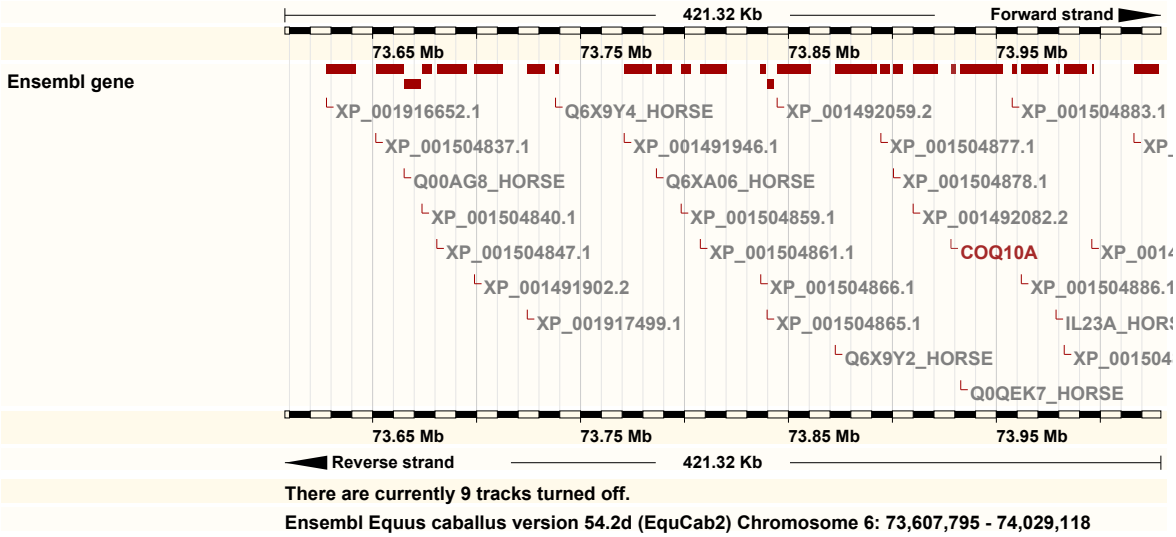
Breeds	Number of horses	Horses with MCOA	Silver colored horses	Non Silver colored offspring
Icelandic horses	237	1	132	65
Rocky Mountain	76	31	61	0
Kentucky Mountain	2	1	0	0
Morgan	8	0	4	3
American Saddlebred	1	0	0	0
Mini	52	0	8	0
Ardenner	3	0	1	2
Swedish Halfblod	2	0	2	0

Table 1, General overview of the material

Extraction of DNA

DNA from hair samples was extracted using the Chelex protocol (Walsh JL. *et. al.* 1991). To approximately five hair-roots 93 µl of 5% chelex solution and 7 µl



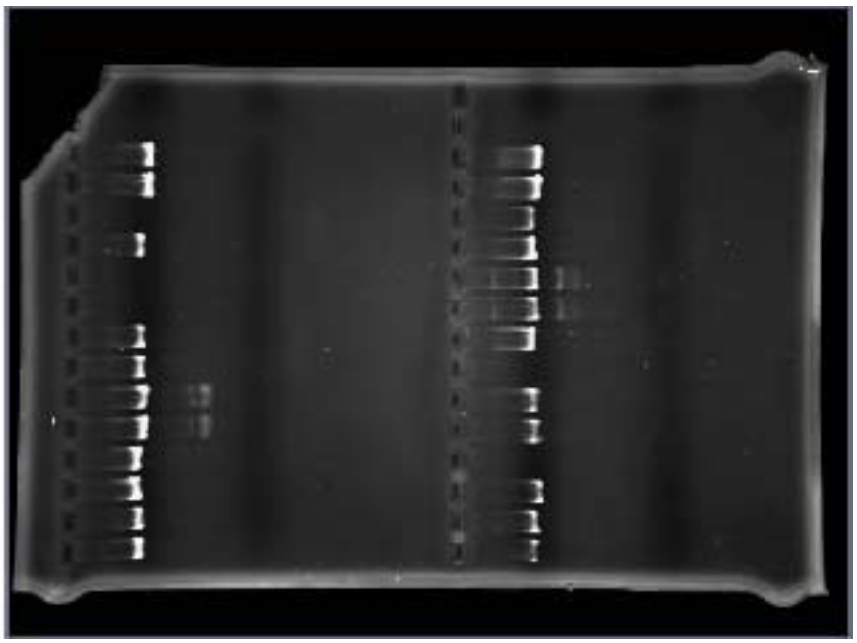


Picture 5. A, Illustration of the original interval at horse chromosome 6 including human synteny. B, Genes in the previous interval. (Ensemble release 54)

Proteinase K was added. The proteinase K reaction was performed one hour at 65°C and then inactivated in 95°C for 10 minutes. The Chelex are magnetic beads that attract the remnant of the cell structures leaving the DNA easily retrievable in the supernatant. DNA from blood were extracted using the Gene Mole® instument and the Mole Strips™ DNA Blood Kit. 100 µl blood was eluated in 200 µl medium according to the manufacturer's protocole.

SNP detection and evaluation

Using the Ecu.Cab 2.0 an additional nine SNPs other than those located in *PMEL17* were detected. In the known interval markers were chosen that were evenly distributed with a distance of approximately 60 kb inbetween. We placed two of the markers outside of the interval in order to obtain clear borders for the haplotypes when investigating the results. These SNPs were investigated via sequencing to evaluate whether or not they were polymorphic. Primers for PCR and sequencing were designed using the primer3plus software (Untergrasser A, *et. al.* 2007). The markers were amplified with touchdown PCR and the products were investigated on a 1.5 % agarose gel in 2 % SDS buffer at 90 Amp. As reference, a 1 kb gene ladder was used. To stain the DNA the gel were left to soak in an ethidium bromide bath for 15 minutes. The gels were UV illuminated and recorded using Bio-Rads Molecular Imager ChemiDoc XRS+ System. For primer sequences of all markers used in this project please view appendix 1.



Picture 6, Control of PCR products by agarose gels.

The PCR products were then dephosphorylated by adding calf intestine alkaline phosphate (CIAP) in preparation for the sequencing. For most markers the forward primer was used for the sequencing but in a few cases the reverse primer

was chosen because of repetitive parts in the sequence of the forward primer. CodonCode aligner 3.0.1 was chosen to analyze the result from the sequencing. The program aligns the sequences allowing for a quick SNP investigation. For those SNPs that turned out to be non-polymorphic, alternatives were chosen in the proximity and investigated in the same way. Appendix 2 PCR, CIAP and sequencing protocol.

Genotyping

In order to receive a high throughput and fast genotyping, the 7900HT fast real-time PCR system from Applied Biosystems (ABI) was chosen for PCR and allelic discrimination in one step. After investigating the sequence using the repeat masker program and the filebuilder 2.0 program, custom made primers and sequence specific probes were ordered from ABI.

Marker Name	Sequence
NI1	AAAGTATGTAATAGCGAGCCCCTGAAGGGGAAGCAGCGTCATAGGT GAGG (G/T)CCAGGTAGTAGTGATTCTGATGCGTAGCTGATTTGAAAA TACA
PMEL17	AGGATTTATTTCAGGTAATGGTAGTCTCCAAGGGAAGACTGGAGAC AAGAGAGGCATTGCTGTCCACACCTGGGTAAATCATGGCATCACAT GAGAGCACTCAGACCTGCTGCCCACTGAGGAGGGGGCTGCTCTCA CCAAAGGGGGAAGAGCGGAAGACCCAGGGCAGACGCAGCCAGTG GGTTCTACCGTGTGGCAGCTGGGGAAGGGGGAGAGCTGAGCCCTG CTTCATAAGTCTGCGCCTGATTTGGGAGAAGAAGAAGGAAACTGGT AAGCAATGGACAAGGGCAGCCTGTCTGTGCTTCTCCAAAGAAGAG GACACACCCACATCCCAGGTTTACTTGGCCTTGATCCTAGCTGCCTC CTCTTCTCCTCACAAACAGGGTTCATTTCTCTTGTTTATATGGTAACC TCTGTCCATTCCCCTCTCACTCTACAAGCTTCCCTGCTTTGAGGACGT
BIEC2-1018345	TCCTGCTCGGAGTCTCTAAAAGCGCTAAGGAGCTCAGTGTCTAGGG ATTTCCAGATTTCTGAAAGGAGAGTGAACAGTCACTGCCAGATCAG GGTGGAATTAAAAGAGGCATGGGTGGGATTAAGGTAAAGGCCCCA AAGGAGAAATTCTGGAGTTGGAGTAGCAAGCAGATAGAAATGGTC ATAAGCTGGAGTTAGATA[C/]GAAATAGAGATGAGAAAGATCTCCTC ATCACCACCACCCCCCATTCTCCTGCCCAGACAAGGTCCCCATGAC TGCCCCTCCTCAGCTGGCCCTAGATCTGTGACATTCTCTAAATGGTT GGAATAGCCAGGAATGTAGGTCAGAGCTAGAGCCAGGGCTTTCTCC TCAATGGCTCCTCCCTCCTCCATCCCCGTCTCCAG

BIEC2-1018377	ACAGAACAACRCTTTATAACATTGCAGGCATTTAGTAKAGTAACTCC ATTTTTGGTACTTTTTAGTATTTTATGTTAGTGGTTTGGATCAGAAAA AACTTTTTTTTCTTCTTGCAGTTTATGGCTTCTGAGGCACTTGTGATC CCAGATACCAGTTTGAAACTTTTCGGTGGAATACCTCTTTGAGATCT GCTAAAAGCT[A/C]TAAGCTATGAGCCTGCTGAAGCCCAGGGACCTT AGATTAAGTCCCCTTGGATTATACACTTGTTTATGAGGAAATAAGTCT ACATAAATTGAAGTTACTAACATAATTGGGGTGTCTGAGAAGGGAA GAAGGTAGCATTTAACAGTGCATCAGCTTCGAGTCAGCATTTCCATG TCTCCTTCCTGTTGTCCCCAACGCCT
BIEC2-1018453	GGGGCAGAGACAAAGCCAGGATCCTGACGTTCTTCTTTTCTTTCAG CTACCAGCTCTGCTTCGTCCTCCTGAGCCCCTCCCTACACTCGATGT GCTCCTGCAGGGGCTGGGCCTGCTGCTGGGGGGCAGCCTCATGCTC ACCATTGCCATGCTGGAGGAGCAGCTATGGCCCCTGGTCTCTGATG GCTGACGGGGGCCAG[T/]GGAAAGGCGTCCAGGTTGCCCTTCCTTC CCCCCAACCACAGGAATGGAGGCGGGACACAGGGCCAGTAGGAG CAATAGGATTTTAATAAACAGAACCCATCCCAAAGCCATGACTATG ACAGTTGTACTTGCACCAAACAGCATAGAAAACCAGGACGTGGTG GGAGGGCTCAAAGCGGTTGGGGGAGGACATGAGTAGG
BIEC2-1018475	CAGTAGTTATAACGTTCTCCAAAACCTCCCTCCTGAGTCCTTCAATT CTCTCTCATATGACCACCTTCCCCGGAACCTTTCCCCATATCCCAGA GGGCTCCCGCTGGTATCCCCCAGCACCTTGCGGTATTTCTTAGAGA CCATGTGGAGATGTGGCTCCTCTTCCCGCCCTATTGGTGACTCAGTG ACCTGGCTGAAGC[AT]GTCAAATTCACTGTCTGACTCCTTGAGTCG GTAAGGAATCTAGAATTTTAAAAAAAGAGGTAAACTTGTTGGATGC CTTAATCCTCTTTGGTTTTCCCCTACCTTTATCCCAACACTGAAACA GCCAGCAAGTCCTGAACCTTCTAATTTCATAGGGTTTCCAGCATCCA CTCCTTCCTTTTTATTCGCATGGGTGTCAC
BIEC2-1184963	GGCAGCTGGTTTCTGAACCCCTTGGGGTCAAATTCCTAAACCAAGA AGATGGGTTGAGTCAGAGGGTTTAATCTTGCCACCGTGGAGCAGGA ATGGGATAGCCTTATCAGCCGAGGCATCTGCCATCATCTGCTCTTCT TCATCCTCATGAAGCAATCCATTAGCTGCCCTGCGCTTGTCCTACCC TCCTTTTCCCACTT[T/C]CCAGAGATCCTAAGGGATTAGCCTTCTGAG CAGGTAGAGAGGAACTTACAGTAGTGAACTTGTGGATAGGGATGCG CTCCTGTCCCTCGGCAATAGTGTAGAAGAGCAGATCACCCAGGCGG GACAGCATGCCACTCTCTGAGGAATCACTGTTTGGGGCAGAGCCGG GGAGGAGGACAGGAACCTGAGAAGTGCTTCCT

Table 3 shows the sequences used to order primers and probes both from Primer3plus and from ABI.

Sequencing of the SMARCC2 gene

When studying the genes in the final interval the *SMARCC2* gene was chosen for sequencing because of its importance during ocular development. This gene contains 29 exons. When investigating the gene using the UCSC genome browser a possible area for the promoter region was detected 890 bp upstream of the first exon. Primers were ordered to cover all the exons, the promoter region and introns 15-20 since they are more conserved than other areas. Five individuals were chosen for the sequencing, three with MCOA and two healthy.

Area covered by primerpair	Direction	Primersequences
Exon 29	Forward	CCAGAACCTGAATCCCCTTT
	Reverse	GAAGGACTTGGGGTTTGTCA
Exon 27 & 28	Forward	CAGAGAGGCCCTTCCTCCT
	Reverse	ATCCCTCCTCAAACCCAGAT
Exon 26	Forward	TGCTGGCTCAGGGATATAACA
	Reverse	ACTCCCTTGCAATGAGAAGC
Exon 25	Forward	TCTAACAAGGGCCAAGCACT
	Reverse	CTCCCTTCACCTCCTTCCTC
Exon 23 & 24	Forward	TGGAAAGGAGAAGACAAGGAGA
	Reverse	TAGTTCAGCTCAGGCAGCAA
Exon 21 & 22	Forward	CTTACTTCGGGGTGATCTGG
	Reverse	CCCCTGCTGGTCTAGATGAA
Exon 20	Forward	CGTCACTTTGGCTGCTTCTT
	Reverse	CTTGGAGATTCCCCTTGGAC
Exon 18 & 19	Forward	GTACTCTGCTGGGTCCAAGG
	Reverse	AAGAGCTGGATGACCTGGTG
Exon 17	Forward	ACTTCCTCCCTTCCTGCTGT
	Reverse	CAAGGGCCACAAGAATCAAC
Exon 16	Forward	AGCCACATAACCTTTCTCCTCTT
	Reverse	TTGGGTGTCACTGGCATTTA
Exon 15	Forward	ACAGGGTTACATGGGACAGC
	Reverse	GGAATGTTTGAACCCCTTGA
Exon 13 & 14	Forward	CCTGTGGGCAAAAATCATGT
	Reverse	TTTTGAGTCCTGGGGTTCAT

Exon 11 & 12	Forward	TAGCAACCCTCTGCCTCTTC
	Reverse	CTGGCACCGAGAAGAATGAT
Exon 9 & 10	Forward	GACTGTCCGTGGTGATGAAA
	Reverse	GATTTCAGCCAAGACATTGACA
Exon 7 & 8	Forward	TAGTTTCCGCCCTTCTTGTC
	Reverse	GGGTGATTCTTGTCTTTCTTTTTC
Exon 5 & 6	Forward	TCAGCCTAAGCCTCATCCAC
	Reverse	TGTACACGTTCCGCTTCTTG
Exon 3 & 4	Forward	CCTGTCACAACTGGGAAACA
	Reverse	TCAGAAAAATGGCTGGGACT
Exon 2	Forward	GGGATCTGGGGCTCACTAAT
	Reverse	GATCTTGGGCAAGCCATTTA
Exon 1	Forward	TGCCCAAGATCACAGAGACA
	Reverse	TTCCATTGCCTGAACTCCAT
Promotor region	Forward	CACACCCCTTTACCCAAGAA
	Reverse	TCGACGAAATCCAAATGACA

Table 2, Sequences of primers used when sequencing the SMARCC2 gene.

Results

When evaluating the markers, nine were found that proved to be polymorphic over the chromosomal interval. These markers are not as evenly distributed as intended since there were some difficulties in finding polymorphic markers in parts of the interval, especially in its 3´end. In this area the sequence seemed to be more homozygous than in the surroundings and it was also highly repetitive. The data from these new markers were combined with the data collected in previous studies to obtain a good resolution of the chromosomal interval.

Marker	Position on ch6	Distance to next	In Kb
TKY 570	66793555	3795804	3795,804
TKY 412	70589359	2955419	2955,419
MS12	73544778	63017	63,017
MS1	73607795	32699	32,699
NI1	73640494	17674	17,674
MS14	73658168	7137	7,137
PMEL17	73665305	57316	57,316
MS3	73722621	3471	3,471
BIEC2-1018345	73726092	23403	23,403
MS7	73749495	18993	18,993
TKY 284	73768488	19523	19,523
BIEC2-1018377	73788011	47073	47,073
MS13	73835084	69868	69,868
BIEC2-1018453	73904952	63230	63,23
BIEC2-1018475	73968182	60936	60,936
MS21	74029118	34130	34,13
BIEC2-1184963	74063248	603761	603,761

MS10	74667009	808225	808,225
MS11	75475234	753330	753,33
MS8	76228564	1522501	1522,501
MS9	77751065	1105381	1105,381
MS4	78856446	616429	616,429
TKY952	79472875	-	-

Table 3, Polymorphic markers used to map the interval. New SNPs are marked in green and the old microsatellite markers are marked in yellow. The borders of the new interval are marked with a thicker line.

When genotyping the horses with known phenotype, two distinctive disease haplotypes could be detected. One shorter at 265 kb and one longer at 420 kb. The disease haplotypes were carried by 96 % of the horses phenotyped with complete MCOA. Of the horses carrying one of the disease haplotypes 85% carry it on both chromosomes. The horses that were merely displaying small ocular cysts 89% carry the disease haplotype and of those 97% were heterozygous. In the group that were phenotyped as nonaffected only 20% carry a disease haplotype and in those cases they were heterozygous.

N1	PMEL17	8345	TKY284	8377	MS13	8453	8475	4963	MS21	
T	T	C	177	C	222	G	T	C	261	Long disease hap
G	T	C	177	C	222	A	A	T	253	Short disease hap

Table 4, The disease haplotypes.

The sequencing of the *SMARCC2* gene exposed one polymorphism where the MCOA affected horses and the healthy controls had different alleles. This polymorphism is located in the intron between exon 19 and 20.

Sample	**Phenotype**	**Genotype**
Is 1	MCOA	CC
G11	Cyst	GC
E11	Cyst	GC
W1	Healthy	GG
O3	Healthy	GG

Table 5, Result of the *SMARCC2* sequencing.

Discussion

The differences in length of the disease haplotypes is caused by the fact that some of the affected horses are carrying a recombinant chromosome. This helped to decrease the interval by 37 %, leaving an interval of 265 kb. One reason for the large decrease was that in the previous study the markers used were microsatellites, and in the downstream end of the interval there were large gaps between the microsatellite markers. By using SNPs in this study, these gaps could be investigated and in a number of markers found in these gaps, there were allelic differences within the group of affected horses. Within the

GENE	KNOWN/EXPECTED FUNCTION
SLC39A5	Encodes a transmembrane protein that is probably involved in zinc transport.
OFC2B	Is a vital part of the DNA damage control system.
RNF41	Acts by regulating degradation of its targets, one of those being the ERBB3.
SMRCC2	Is an important part of five chromatin structure remodeling complexes. Has been proved to influence ocular development in other species.
MYL6B	Myosin light chain contributing to the sliding between filaments during muscle contraction.
FAM62A	A transmembrane protein that is probably activated by Ca^{2+}. The Human homolog is extresses in the entire organism but enriched in the brain.
ZC3H10	As a protein with a zinc finger domain the function of the protein is most likely to be regulation of transcription.
Pa2G4	The biological function of the protein is probably negative regulation of transcription.
ERBB3	A tyrosine kinase that is very important during cellular development and differentiation. In a number of human diseases, causative mutations have been detected in ERBB3
RPS26	Is a ribosomal protein and thereby important for translation.
IKZF4	A DNA binding protein that interacts with MITF andSPI1. Is expressed primarily in muscular tissue and may be involved in neuronal development.
SOUX	Sulfide oxidase that is crucial for normal function of the mitochondria. Lack of SOUX protein lead to irregular brain development and often early death.
RAB5B	A cell membrane protein that is involved in protein transport.
CDK2	A phosphorylating protein that is activated during the S and G2 part of the mitosis.
PMEL17	A protein important for the formation of eumelanin. Has been proven to be down regulated in developmental disorders in other animals.
DGKA	A part of the intracellular signal transduction following activation of a G-protein coupled receptor.

Table 6, Genes in the resulting interval and known/predicted protein function. (Ensemble 53)

remaining interval there are sixteen genes still remaining to be investigated, some of which are more likely to be involved in the development of the eye.

SMARCC2 is located in the 5′ end of the present interval, approximately 179 kb from the *Pmel17* mutation. It is an actin dependent DNA binding molecule that is a vital part of the SWI/SNF chromatin regulating complex (Wang W, *et. al.* 1996). This complex has been proven to influence cellular differentiation in a number of tissues. The lentil tissue and the retina is an example of tissues whose normal development is dependent on the SWI/SNF complex (Duncan B & Zhao K, 2007; Das A, *et. al.* 2007). In many types of cancer the *SMARCC2* gene is up-regulated and functions as an oncogene (Naidu S, *et. al.* 2009). These facts make it a very interesting candidate for the *MCOA* locus and are the reason why this gene was chosen for sequencing. The genomic sequences of three horses with MCOA and two healthy controls were compared. At one allele the samples were different from the controls. This allele is located in the intron between exons 19 and 20. This area is not conserved (conservation score 0, 66 according to UCSC, phyloP44wayPlacMammal) something that contradicts the possibility of this being the causative mutation. Therefore this intron should be investigated in a larger number of horses to establish if the polymorphism is completely corresponding to the phenotype of the horse. If it is not then it may be used to further decrease the interval.

A strong candidate gene to be the cause of the *MCOA* phenotype is still the pre-melanosomal protein 17 *PMEL17.* The PMEL17 protein is vital for the formation of the eumelanosome (Chakraborty A, *et. al.* 1996). The PMEL17 protein is cleaved and then polymerizes to form a grid within the melanosome upon where the eumelanin granulates assemble. The *PMEL17* mutation, which has been detected as the origin of the Silver dapple coat color, is a missense mutation in exon 11. This lead to a change of the amino acid sequence of the proteins cytoplasmic region (Brunberg E, *et. al.* 2006). The melanosomal pathway is also important in the embryonic development and differentiation. *PMEL17* mutations can be found in a number of species for example mouse and dog, giving a phenotype of diluted eumelanin coat color. In some cases the animals with this phenotype also display various phenotypes of developmental distortion. In dogs the coat color blue merle is caused by a mutation in *PMEL17.* Among dogs with this coat color the prevalence of auditorial impairment is higher (Clark L, *et. al.* 2006). In zebra fish the dilution of eumelanin leads to distorted ocular development (Schontaler H, *et. al.* 2005). In this dataset the *PMEL17* SNP matched perfectly to the disease phenotype.

Erythroblastic leukemia viral oncogene homolog 3 (*ERBB3*) is a membrane bound epithelial growth factor (Kraus M, *et. al.* 1989). It binds to neuregulin and forms a heterodimer with other members of the Erb family. The kinetic activity of the heterodimer activated a number of pathways important for cell proliferation and diversification. This is the reason why the gene is upregulated in a variety of cancer types (Chakraborty S, *et. al.* 2007). The reason for *ERBB3* to be a strong candidate to cause the *MCOA* phenotype is because of its importance in cellular development. This may influence the development of the eye. There are also some indications that the Erb group is influencing the expression of *PMEL17* due to the short distance between the loci. The third candidate for *MCOA* is the ikaros family zinc finger 4 (IKZF4). This is a DNA binding transcription factor that down-regulates transcription of genes involved in bone development (Perdomo J & Crossley M 2002). The reason for it to be a candidate for *MCOA* is that the molecule that the IKZF4 interacts with is the transcription factor MITF (Hu R, *et. al.* 2007). This complex is the major regulator of *PMEL17* transcription and if this complex is disrupted this may influence the entire downstream interactions.

In this study the phenotyped horses were divided into three groups. The horses of the first group had a number of ocular abnormalities, full-blown MCOA. The second group constituted horses that only showed small ocular cysts and the third group of horses were those where no ocular abnormalities had been detected. There were clear genotypic differences between the three groups. In the MCOA group, 89% were homozygous for the disease haplotype while among the horses in the cyst group 93% were heterozygote for the same haplotype. Among the horses that were phenotyped as healthy 24 % proved to be heterozygote for the disease allele. These horses may very well have small cysts since these can be difficult to detect. This result is consistent with the hypothesis of the inheritance pattern being codominant with incomplete penetrance. There is still much work left to be done in order to find the causative mutation for MCOA. The candidate genes must be further investigated both genetically and functionally. When the locus has been found then the breed associations that are affected may develop breeding programs that avoid mating carriers with each other.

Conclusion

The primary aim of this project was primarily to diminish the interval wherein to look for the *MCOA* locus. The resulting interval is approximately 37% shorter than the original. Most important is that there are now fewer genes to investigate. The gene chosen to be sequenced in this project, the *SMARCC2* gene, hold an interesting polymorphism that needs to be explored further. Finding the causative mutation and studying the function of the protein may not only help animal breeders to generate a healthier stock but may also evolve our understanding of the complex epigenetic regulation during ocular development.

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Appendix 1

All Markers tested

Marker name	Position	Forward	Reverse
BIEC2-1018319	73640354	GAAAAGGTGGGGGAAATAGC	TGGCATGCAATTACAGCATT
BIEC2-1018290	73545965	AGGGAGTTGCCTTTGTTCCT	TGTGGATTTGCTGGTGGTTA
BIEC2-1018299	73568548	GCTGGGGAAAATTTGAAAAA	GTGCTTCAGGATGGGAATGT
BIEC2-1018307	73597316	CGGGGTTAGAATCTGTGCTC	GGAACCCAACTACCCCCTAA
BIEC2-1018309	73604541	TTAAGAACCAGCTCCCCTCA	GAGCTGAACTCAGAGCCACA
BIEC2-1018318	73640017	GCAGCGAGGAAATGGAAATA	TACAGCACATGGCAGGAGAA
NI1	73640494	GAAAAGGTGGGGGAAATAGC	TGGCATGCAATTACAGCATT
BIEC2-1018320	73640624	GGGCAGCATCAAAGGTAATC	TGGTACAACCTCTTCTCCTGCT
PMEL17	73665305	CCTGGGTAAATCATGGCATC	GTGAGAGGGGAATGGACAGA
BIEC2-1018345	73726092	AAGCGCTAAGGAGCTCAGTG	CATTCCTGGCTATTCCAACC
BIEC2-1018377	73788011	TGTTAGTGGTTTGGATCAGAAAAA	GGGACAACAGGAAGGAGACA
BIEC2-1018423	73857478	CAGAATCAATGGGACGGAGT	TCTTCTGCACTGGGGCTACT
BIEC2-1018423	73857478	TTTCTTCCCAGTAATGTTTTTCTGT	TCCTCAGTCTTGGTGGAGGT
BIEC2-1018424	73858845	CATGGGAAACGTAAAGTGGA	GTGAGTGGATGGGTCAGGAG
BIEC2-1184873	73860926	TGGGAGACAGAGGAAGGAGA	GGTTGAGGAAACAGGGTCAA
BIEC2-1184882	73871285	AGTGAGGGTGACGACAGGAG	TTAACATAGGCAGGGCCAAG
BIEC2-1018453	73904952	TACCAGCTCTGCTTCGTCCT	TACTCATGTCCTCCCCCAAC
BIEC2-1018454	73913915	GGGCAGTGAGAAGTCTAGGG	CGCTGGTGAATCTGGATGTA
BIEC2-1018475	73968182	TTCTCCAAAACCTCCCTCCT	GGATGCTGGAAACCCTATGA
BIEC2-1018509	74029403	GGAGTCAGCATGTCCTCCTC	TCCCCATCTTCCTTTGGTAA
BIEC2-1184963	74063248	CCCTTGGGGTCAAATTCCTA	TCAGAGAGTGGCATGCTGTC

Appendix 2

PCR, CIAP and Sequencing protocol.

PCR x	1	4	10
H20	16,4	65,6	164
Mg	2,5	10	25
Buffert	2,5	10	25
dNTP	0,3	1,2	3
Taq	0,3	1,2	3
F primer	0,5	2	5
R primer	0,5	2	5
	23		
DNA	2		

Reaction mix						
Number:	**1**	**50**	**20**	**25**	**40**	
Vol. PCR:	25	25				
CIAP	0,05	2,5	1	1,25	2	
Exo1	0,1	5	2	2,5	4	
Exo1 buffer	2	100	40	50	80	
Tot	2,15	107,5				
Mix -> 37°C, 1h -> 85°C, 15min-- GabSEQ på PTC2000						
Sekvensering				**Primrar**		
späd ca 5 ggr (till ~100ng/ul)				Start c= 100uM		
				späd 10 ggr (10+90) som vid vanlig PCR		
27	PCR o CIAP mix			Späd 5ggr (10+40)		
108	H_2O					
135						
På plattan						
12 ul vatten						
4 ul PCR och CIAP						
2 ul Primer						

Allelic discrimination					
384 platta, dvs 5 ul per brunn					
	1	**96**	**110**	**384**	**420**
Master mix	2,5	240	275	960	1050
Assay mix	0,125	12	13,75	48	52,5
DNA ca 4 ng/ul	2,375				