Institutionen för husdjursgenetik

Mapping of the silver coat colour locus in the horse

by

Emma Brunberg

Supervisors:
Gabriella Lindgren, UU
Sofia Mikko, SLU

Examensarbete 279
2006

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).

Institutionen för husdjursgenetik

Mapping of the silver coat colour locus in the horse

by

Emma Brunberg

Agrovoc: Horse coat colour, silver dapple, PMEL17, genetics

Supervisors:
Gabriella Lindgren, UU
Sofia Mikko, SLU

Examensarbete 279
2006

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).

Contents

Abstract	1
Introduction	1
The silver dapple colour	2
Linkage analysis	3
SILV as a candidate gene	3
The present thesis	5
Materials and methods	5
Genotyping of genomic DNA using microsatellite markers	5
Linkage analysis	5
Sequencing of *SILV*	6
SNP analysis using pyrosequencing	6
Results	7
Genetic markers and genotyping	7
Linkage analysis	7
Polymorphism discovery by DNA sequencing	7
Association of DNA polymorphism and the silver phenotype	8
Discussion	8
Acknowledgements	9
References	10
Pictures	12
Figures	13
Tables	14

Abstract

The silver dappled colour in horses is controlled by a dominant allele that dilutes the black pigment eumelanin. A black or brown horse that carries the allele becomes diluted in mainly mane and tail, while the hair of the body remains dark. The silver dapple colour is common in the Icelandic horse population and has also been observed in for example Shetland pony, Norwegian nordland, Rocky Mountain pony and Ardenne.

The purpose of this project has been to try to map and characterise the silver dapple coat colour locus in the horse genome. This has been done by performing a linkage analysis with markers throughout the genome and a marker near a candidate gene; *SILV* at *Equus Caballus* chromosome 6 (ECA6). *SILV* is coding for a protein called Pmel17 that is involved in the production of eumelanin. The gene has been shown to control colour-diluting processes in mouse, chicken and dog. A half-sib family consisting of one Icelandic stallion, 34 of his offspring and 29 of their mothers were used (five offspring shared mother with another offspring). Seventeen of the offspring had the silver colour. Significant linkage was found between the silver phenotype and the marker TKY284 near the *SILV* gene. The six last exons and introns were sequenced and SNPs were found in intron 9/10 and in exon 11. The nucleotide substitution in exon 11 changes the second amino acid in the cytoplasmic region from an arginine to a cysteine and the same mutation is found in one type of hypopigmented chicken. Twenty-five silver horses from three breeds and 55 non-silver individuals were genotyped for the mutation and this showed a full association with the phenotype. These data shows that *SILV* probably is responsible for the silver dapple phenotype in horses. The cytoplasmic region of the protein is well conserved among vertebrates. The remaining parts of the *SILV* gene will be sequenced to search for more mutations and the mutation in exon 11 will be transfected into cell-lines to see how it affects the protein function. Of the pigment genes that earlier have been cloned, a majority is associated with human hereditary pigment diseases. Identifying genes and mutations for a pigmentary disease or a pigment phenotype can increase the understanding of the function of the protein and the molecular mechanisms behind the process of pigmentation and pigmentary changes.

Introduction

Coat colour is a trait with a great variation among our domesticated animals. Breeders and animal owners have always been affected by and paid attention to coat colour variation. The colours have been surrounded by rumours that a certain quality is associated with a colour and these animals can be more or less economically valuable. The knowledge of how certain genes and their gene products affect coat colour is of great interest for basic research. In mouse, around 125 known loci are known to affect pigmentation if mutated. Some coat colours are associated with diseases, for which it is necessary to know the underlying functions of the coat colour gene to be able to treat these pigmentary disorders. Some coat colours are difficult to distinguish from others and molecular genetics can provide DNA-tests for these colours.

Melanin is the pigment that is essential for the colour of skin, hair and eyes. The pigment is synthesised by melanocytes, which are cells originating from the neural crest (Passeron et al., 2005). The pigment is synthesised in special organelles, the melanosomes. Melanins are derivates of L-DOPA that are derived from α-tyrosine or α-phenylalanin, a reaction that can be catalysed by an integral membrane melanosome constituent, tyrosinase (Slominski et al., 2004). There are two types of melanin, eumelanin and pheomelanin. The two reactions that produce the two pigment types are different. The production of

pheomelanins (red pigment) involves reduction reactions and the production of eumelanins or black pigment, involves oxidation and hydroxylation reactions that will result in reactive indoles. If these indoles are exposed to components in the cells they could be deleterious and that is why the reactions require melanosomes (Raposo and Marks, 2002).

The silver dapple coat colour

In horses there are three basic colours; Black, bay and chestnut: The bay and chestnut horses can vary from relatively light to darker shades of the same colour. For the black colour, the horse has to have the E-allele at the extension locus. This is a dominant allele and therefore all *E*- -horses will be able to produce black pigment (Furugren, 2002). Horses that are black or bay as a basic colour will be E- at the extension locus (Furugren, 2002). Another locus, *A* (agouti), will control the distribution of black and red areas on horses that can produce black pigment and can restrict the black pigment to certain parts of the body (Sponenberg, 2003). This means that a black horse will carry the recessive alleles *aa* and a brown or bay horse will be *A*- at the Agouti locus. A chestnut can carry any allele at this locus, but will still show a real phenotype because of the lack of black pigment (i.e. a chestnut horse will always be *ee* at the extension locus) (Furugren, 2002).

Many of the colour variants that derive from the basic colours are diluted. Those colours often fascinate breeders and are often very popular. There are four categories of the diluted colours: linebacked duns, cream-related colours, champagne and silver dapple (Sponenberg, 2003). The silver dappled colour in horses is controlled by a dominant allele that dilutes the black pigment eumelanin. A black or brown horse that carries the allele becomes diluted in mainly mane and tail, while the hair of the body remains darker. The genetically black horses are diluted to dark brown or almost black colour with silver grey or white mane and tail. The genetically bay or brown individuals are diluted to a lighter brown or almost chestnut-like colour with silver grey or white mane and tail. The silver brown individuals can be hard to distinguish from a chestnut horse with flaxen mane and tail, but it often has a darker shade on the legs (Bowling, 1996) and lighter eyelashes (See Picture 1 and 2). In some countries and some breeds one distinguishes between a large variety of silver variants that are believed to depend on the basic colour of the horse. For example, the bay individuals are thought to be the ones that gives the typical "red silvers" while the brown silvers are believed to have a darker brown shade as a basic colour (Sponenberg, 2003). Chestnut horses can carry the silver allele and inherit it to the offspring, but will not be affected in colour because the gene only affects the black pigment. This means that the silver allele will only change the phenotype on *E*- - individuals. The silver allele is assumed to be fully dominant, i.e. silver heterozygotes and homozygotes are indistinguishable (Furugren, 2002).

The silver dapple colour is common in the Icelandic horse population and has also been observed in for example Shetland pony, Norwegian nordland, Rocky Mountain pony and Ardenne. The reason for the presence of the silver coat colour in Icelandic horse, Shetland pony, Norwegian Nordland and Rocky Mountain is probably due to connections between Norway, Iceland and Great Britain during the colonisation of Iceland. The silver locus in the Swedish Ardenne horse comes from Belgium and therefore it is possible that the mutation causing the silver colour has arised more than once (Furugren, 2002). The colour has also been registered in Mountain Pleasure Horse, Kentucky Mountain Saddle Horse and Arabians. Silver dappled horses could also be present in several other breeds, but are likely to be inaccurate identified and therefore not recorded (Sponenberg, 2003).

In some breeds, several silver horses have ocular abnormalities, varying from minimal to quite severe eye defects. The defect is not properly documented but some researchers believe that it is a part of the gene action at this locus and that homozygotes are more severely affected than the heterozygotes (Sponenberg, 2003). In many breeds, however, there is no problem with eye defects among the silver dappled individuals. The ocular defect could

therefore be a founder effect; i.e. the silver dappled colour in the Rocky Mountain pony comes from a family that has a problem with this eye defect.

Linkage analysis

Each somatic cell contains two similar versions of one chromosome, one from the father and one from the mother. The gametes contain only one copy of each chromosome (Alberts et al., 2001). There are two types of cell division: mitosis and meiosis. Mitosis forms two genetically identical cells from one single cell and occurs during normal growth and differentiation. Meiosis is the production of gametes or germ cells. The gametes are formed from one diploid cell (e.g. containing two copies of the same chromosome) that will undergo replication and two cell divisions (Liu, 1998). A chromosome will replicate into two identical sister chromatids, and it will therefore be four copies of each chromosome. The sister chromatids are paired with its duplicated homologue and form a bivalent. This will then be followed by two cell divisions resulting in four haploid gametes (Alberts et al., 2001). Within the bivalent, the chromatids can undergo physical breakage and exchange of DNA segments, this is called recombination. If no recombination occurs, the linkage is complete and if a certain amount of recombinants occur the linkage is said to be partial (Brown, 2002).

When performing a linkage analysis one wants to follow the segregation of alleles in the pedigree. Loci on separate chromosomes will recombine freely, whereas two loci situated near each other recombine at a much lower rate. With a low recombination fraction linkage is indicated and with a high Lod score this is verified (Marklund, 1997). The recombination fraction is the ratio of recombinant gametes and total gametes, e.g. if the parental types of one loci are AB and ab; Ab and aB are recombinant types (Liu, 1998). The Lod score is a measure of how likely the observed family data are assuming linkage versus assuming free recombination. The standard significance thresholds for Lod score (Z) is $Z>3$ for linkage and $Z<-2$ for exclusion of linkage. Testing more offspring in the same family and also add data from different families, will increase the statistical power (Marklund, 1997).

To be able to perform a linkage analysis one needs to have genetic markers. Today, DNA polymorphisms distributed over the whole genome are used for this purpose (Marklund, 1997). An important feature for a marker that should be used in linkage analysis or genetic mapping is that it shows polymorphism. There are three major types of DNA polymorphisms: restriction fragment length polymorphisms, simple sequence length polymorphisms and single nucleotide polymorphisms (Brown 2002). One of the most used markers are microsatellites, which are quickly analysed, abundant in the genome and have a high degree of polymorphism (Marklund, 1997). Microsatellites are tandem repeat sequences with normally mono- di-, tri- or tetra repeats. Due to the variation in the number of repetitive units, the marker exhibits its high level of polymorphism (Ellegren, 2004).

Different types of pedigrees can be used for linkage analysis. In the ideal case, the segregating generation has 100% heterozygosity and this is derived when crossing for example divergent breeds. It is very time consuming to make such pedigrees in animals with a long generation interval, therefore one often uses half-sib families from available family material. One of the parents has to be double heterozygote at the loci investigated (Marklund, 1997).

SILV as a candidate gene

The gene product of *SILV/Pmel17* has an important role in melanogenesis. Melanosomes that produce eumelanin go through four maturation stages. The first two stages generate a matrix consisting of intralumenal striations that are composed of fibrillar material. In the two later stages of maturation melanosomes are deposited on the matrix and will then blacken (Raposo and Marks, 2002). The major polypeptide in the matrix is pre-melanosomal protein (Pmel17), the product of *SILV/Pmel17* (Kobyashi et al., 1994). Except from being a component of the

fibril matrix, Pmel17 is also important when this is formed. This protein, together with other known melanosomal proteins are proteolytically cleaved in the stage I melanosomes. For many other proteins this inhibits the catalytic function, but for Pmel17 this cleavage is essential for the protein to change from a membrane-bound to a free form that can be a part of the fibrillar matrix (Kushimoto, 2001). Pmel17 could also have further features important for the melanin synthesis, as protecting the pigment cells from toxic intermediates (Berson et al., 2001).

The silver gene was first identified in mouse, where it was shown to be involved in a recessive coat colour dilution that affects black pigment (Dunn and Thigpen, 1930). The human cDNA of the gene was called Pmel17 and has been shown to be expressed in melanocytes but not in non-pigmented cells (Kwon, 1991). The gene in human has been sequenced and mapped to human chromosome 12, the corresponding gene in mouse is situated on mouse chromosome 10 (Kwon, 1991). The human gene (SILV, OMIM: 155550) consists of 11 exons and is around 11.8 kb long (see Figure 1) (Bailin et al., 1996), in other mammals the gene is between 7.4 and 13.7 kb long and has between 11 and 13 exons (Ensembl). Researchers have sequenced and isolated Pmel homologues in rat (XP_343147), horse (AAC97108, Rieder et al. 2000), cow (XP_582778, Kim and Wistow, 1992) dog (XP_538223), chicken (AAT58245, Kerje et al, 2004), quail (AAS12180), Xenopus (AAH77508, AAH75473), zebrafish (AAT37511) and *Tetraodon nigroviridis* (CAG11762).

The gene product consists of one N-terminal signal sequence (Maresh, 1994), lumenal domain N-terminal region (NTR), polycystic kidney disease region (PKD) (Bycroft et al., 1999), one repeat domain (RPT) (Kwon et al 1991), cleavage site (CS) (Berson, 2003), kringle-like domain (KLD), Trans membrane spanning domain (TM) and a cytoplasmic domain (CYT) (see Figure 2). The most conserved regions are the cytoplasmic domain, polycystic kidney disease, kringle like domain and N-terminal signal sequence (Theos et al., 2005).

The human *SILV*-gene codes for at least three different isoforms, which have been confirmed with reverse transcriptase-PCR. This will lead to a difference in 21 bp and 7 amino acids, where the major form is the longer one (Bailin et al., 1996). Another form lacks 42 bp and can occur together with the other (Nichols, 2003). Mice with the silver coat colour (*si/si*) have been shown to have nine nucleotide substitutions and one insertion compared with the wild type. The substitutions do not seem to be harmful to the function of the gene. The insertion is predicted to shift the reading frame and create a new termination codon in the cytoplasmic domain that extends the protein by 12 amino acids. This is therefore thought to be the mutation causing the silver coat colour in mice (Martinez-Esparza et al., 1999). In chicken, the homologue is called PMEL17 and causes different diluting colour variants. The colour variants are probably due to dominant insertions/deletions in rather well conserved regions of PMEL17, like the transmembrane region and cytoplasmic region. The different colour variants in chicken (dun, smoky and dominant white) also carry a large amount of other polymorphisms in the gene. All colour variations dilute the black pigment in different amounts (Kerje et al., 2004). It is also possible that mutations in PMEL17 in chicken can decide the pecking order (Keeling, 2004). In dog, the merle phenotype has recently been associated to *SILV*. The merle dogs have patches of diluted pigment and the colour is inherited in an autosomal dominant way. Dogs with this phenotype have a short interspersed element insertion in the border between intron 10/exon 11, which seems to be responsible for the lack of pigment, and also a number of deletions in the oligo(dA)-rich tail of the SINE . Many merle dogs have ocular abnormalities and auditory dysfunction. Individuals that are homozygote for the mutation also have problems with skeletal, cardiac and reproductive systems (Clark et al., 2006). In human, there is no known mutations in *SILV*, but it is believed that the gene could be linked to some forms of albinism (Kwon et al., 1996) and red hair (Kerje et al., 2004). Some researchers also believe that the protein could be involved in

some forms of Waardenburg sha syndrome (WS), which is an auditory pigmentation disorder in humans. The symptoms of WS is similar to the one connected to the merle phenotype in dogs (Clark et al., 2006).

The present thesis

The purpose of this project has been to try to map and characterise the silver dapple coat colour locus in the horse genome. This has been done with a genome scan using microsatellites and by investigating at a marker near an interesting candidate gene. By doing a linkage analysis, conclusions can be drawn about the locus coding for the colour.

Materials and methods

Genotyping of genomic DNA using microsatellite markers

Markers already mapped and known to be evenly spread throughout the genome were analysed to find linkage with the silver phenotype. By choosing microsatellites spread over the genome, there is a chance to find markers that is situated near the locus of interest. By using mapped markers, it is possible to draw conclusions about the chromosomal location of the silver locus when linkage is found. As mentioned above, microsatellites are the type of markers that are most widely used because of the high level of polymorphism between individuals, the abundance in the genome and ease to type.

A pedigree consisting of one half-sib family with one Icelandic stallion, 34 of his offspring and 24 of their mothers, were used (some offspring were full siblings). Seventeen of the offspring had the silver dapple colour. From a panel comprising 102 microsatellite markers spread over the horse genome, 49 were initially used. The sire of all offspring was first genotyped to test if he was informative, i.e. heterozygous for each marker. The markers for which the stallion was heterozygous were then tested in the half-sib family (Table 1). PCR was used to amplify the microsatellites. The amplification was performed in a total volume of 10 µl containing 3 pmol of each primer, ~ 20-40 ng of template DNA, 0.24 mM dNTP, 0.75 units of AmpliTaqGold polymerase (Applied Biosystems), 2.5 mM $MgCl_2$ and 1xPCR buffer (Applied Biosystems). The amplification included 8 min at 95°C, followed by 35 cycles with 30 s at 95 °C, 30 s at annealing temperature between 56 °C and 60 °C (for annealing temperatures see Table 1) and 30 s at 72 °C, ending with a extension step at 72 °C for 7 min.

One additional marker, TKY284, near the candidate gene *SILV* was chosen. The reason for also investigating candidate genes is that this can give faster results compared to a complete genome scan. All horses in the family were genotyped also for this marker. This microsatellite needed different PCR conditions as well as PCR profile. It was amplified in a total volume of 10 µl containing ~20-40 ng of template DNA, 0.20 mM of dNTP, 0.6 units of AmpliTaqGold polymerase, 2.5 mM $MgCl_2$ and 1xPCR buffer. The amplification included 2 min at 95 °C, 5 cycles with 30 s at 94 °C, 30 s at 60 °C and 30 s at 72 °C, followed by 35 cycles with 30 s at 94 °C, 30 s at 57 °C and 30 s at 72 °C, ending with 10 min at 72 °C. The reactions were run in a 2720 Thermal cycler (Applied Biosystems). All microsatellites were first visualised by agarose gel electrophoresis. The markers were then genotyped using the MegaBACE genotyping kit (Amersham Biosciences, Uppsala, Sweden) and electrophoresed using the MegaBACE 1000 capillary instrument (Amersham Biosciences). The analyzing of the electrophoresis was performed with Genetic profiler software (Amersham Biosciences).

Linkage analysis

Linkage analysis for all microsatellites was performed using the LOD-score method within the program CRI-MAP (Green et al., 1990). Two-point analysis was used for testing pair wise linkage between all markers.

Sequencing of SILV
For the sequencing of *SILV,* four horses were chosen, one Icelandic silver stallion that was thought to be homozygous at the silver locus, one silver dappled Icelandic horse heterozygous at the locus and two non-silver Icelandic horses. At some parts of the gene, two more horses were chosen (one silver dappled and one non-silver) to increase the sequence quality needed for SNP discovery.

Primers for sequencing *SILV* were first chosen in the exons that were already sequenced (5-11) (AAC97108) (Rieder et al. 2000). Initially four primer pairs were chosen (Exon 5-6, Exon 6-7, Exon 7-10 and Exon 10-11 according to Ensembl) and when sequence from the introns was achieved another four pairs were designed (Exon 6-7(2), Exon 7-Intron 8, Exon 9-Intron 10 and Intron 10 –Exon 11). All primers were designed with Oligo 5.0 (Molecular Biology Insights) and Primer 3 (Whitehead Institute for Biomedical Research). For primer sequences see Table 1. PCR was used to amplify the gene. The amplification was performed in a total volume of 25 µl including 7.5 pmol of each primer, ~30-50 ng of DNA template, 0.2 mM dNTP, 1.9 units AmpliTaqGold polymerase (Applied Biosystems), 1.5 mM $MgCl_2$ and 1xPCR buffer. The amplification included 10 min at 95 ^{o}C, 5 cycles of 40 s at 94 ^{o}C, 40 s at 61/57 ^{o}C and 2 min at 72 ^{o}C, 35 cycles with 40 s at 94 ^{o}C, 40 s at 57/55 ^{o}C and 2 min at 72 ^{o}C, ending with an extension step for 15 min at 72 ^{o}C. All PCR-products were visualized with agarose gel electrophoresis at 90W in 1xTBE buffer. The PCR-products were sequenced using MegaBace sequencing kit (Amersham Biosciences, Uppsala, Sweden) and electrophoresed with MegaBace 1000 capillary instrument (Amersham Biosciences, Uppsala, Sweden). The sequences were analysed using the Sequencher 3.1.1 software (Gene Codes, Ann Arbor, MI).

SNP analysis using pyrosequencing
Pyrosequencing is a faster way to analyse sequences and polymorphisms than a traditional sequencing procedure (Brown, 2002). In the case where one wants to analyse a SNP a PCR-reaction is performed, where one of the primers is biotinylated. The biotinylated part of the sequence is then immobilised to streptadivin coated sepharose beads. After this the DNA is denatured (single stranded) and a sequencing primer is attached to the single stranded DNA. During the analysis dNTPs are added and the order in which the nucleotides are incorporated are detected. The detection is possible because the addition of a certain nucleotide will release one pyrophosphate, which will be transformed to one ATP with the help of sulifurylase. The ATP will then be converted to light by the enzyme luciferase (Ronaghi et al., 1998). The order of which the nucleotides are added is known and if a nucleotide is not incorporated into the polynucleotide, it will be degraded. This will make it possible to interpret the pyrogram correctly (Brown, 2002).

The most interesting SNP was analysed using pyrosequencing. Animals used were offspring and mothers in the earlier mentioned family, one homozygous Icelandic stallion (also an offspring of the stallion) and two silver dappled Swedish warmbloods, mother and offspring, three silver Icelandic horses outside the family and one silver Ardenner horse. Twenty-six non-silver horses from different breeds (Welsh pony, Connemara pony, Icelandic horse, Thoroughbred, Norwegian fjordhorse, Haflinger, New Forest pony, and North Swedish horse). Three primers were designed, one biotinylated forward PCR primer, one un-biotinylated reverse PCR-primer and one reverse sequencing primer flanking the SNP of interest. For primer design, see Table 2. The PCR was performed in a total volume of 25 µl containing 5 pmol of each primer, ~10-40 ng of template DNA, 0.24 mM of dNTPs, 1.9 units AmpliTaqGold polymerase, 1.5 mM $MgCl_2$ and 1xPCR buffer. The amplification started with a denaturation step at 95 ^{o}C for 5 min. This was followed by 5 cycles with 95 ^{o}C for 30 s, 61 ^{o}C for 30 s and 72 ^{o}C extension for 30 s, 35 cycles with 95 ^{o}C or 30 s, 59 ^{o}C for 30 s and 72 ^{o}C

for 30 s. The amplification ended with an extension step at 72 °C for 10 min. All PCR-products were visualised with agarose gel electrophoresis at 90W in 1*TBE buffer.

To immobilise biotinylated PCR-products to streptavidin beads, 2 µl of sepharose beads and 38 µl of 2X binding-washing buffer (5 mM Tris-HCl, 1 M NaCl, 0.5 mM EDTA, 0.05% Tween 20) per sample were mixed with 15 µl of PCR-product and immobilised in room temperature for 10 minutes. The immobilised PCR-product was then washed once in 70% EtOH. Single stranded DNA was obtained by incubating the immobilised PCR-product in 0,2 M NaOH for around 1 minute and washing the beads once in washing buffer (10 mM Tris-Acetat). To hybridise the sequencing primer to the single stranded DNA, 4 pmol of sequencing primer and 11.96 µl annealing buffer (20 mM Tris-Acetate, 2 mM $MgAc_2$) was incubated with the single stranded DNA at 80°C for 2 min. The pyrosequencing was then carried out with a PSQ96 instrument (Amersham biosciences) and the SNP Reagent kit (dNTPs, enzyme mixture (DNA polymerase, ATP sulfurylase, luciferase, and apyrase) and substrate mixture (APS and luciferin), (Pyrosequencing AB, Uppsala, Sweden).

Results

Genetic markers and genotyping

Out of 49 markers tested, the stallion was heterozygous for 22 of the markers. This corresponds to an observed heterozygousity (H_0) of 44% (See Table 1). All horses in the pedigree had earlier been genotyped for 19 microsatellites (HMS7 at ECA1, ASB17 at ECA2, ASB23 at ECA3, HMS6 and HTG7 at ECA4, AHT5 at ECA8, HMS3 and HTG4 at ECA9, HMS2 at ECA10, ASB2, HTG6 and HMS1 at ECA15, HTG10 at ECA21, AHT4 at ECA24, VHL20 at ECA30, LEX3 at ECAX, UCDEQ425 at ECA28 and COR018 at ECA25) and 10 blood group systems and protein polymorphisms (A, C , D , P, Q, Tf, Es, Al, PGM and Alb) This means that that the stallion was tested for in total 79 markers and he was heterozygous for 42 (54%), covering 25 chromosomes, which then could be used in the linkage analysis.

Linkage analysis

The result of the linkage analysis was obtained using the twopoint test in CRI-MAP. The test of the markers showed three significant linkages. TKY284 at ECA6 linked to the silver phenotype with a recombination frequency of 0.0 and a LOD score 9.0, ASB23 at ECA3 to Albumin protein marker at ECA3 with a recombination frequency of 0.05 and LOD score 4.6 and finally COR075 at ECA15 and HMS1 at ECA15 showed a recombination frequency at 0.09 and a LOD score at 3.0. During the linkage analysis there was a problem with non-inheritance for some of the markers, but these results were corrected or discarded.

Polymorphism discovery by DNA sequencing

In total, 2.65 kb of the *SILV* gene was sequenced, spanning over the six last exons. The six last introns and exons are, with two exceptions (the middle part of intron 6 and the last part of exon 11) fully sequenced. The obtained sequence from both silver and non-silver horses of exon 6 differs from the published sequence at several places, which will also change the amino acid sequence. In the sequenced region, two polymorphisms that could be linked to the silver phenotype were found. Of those, one is a missense mutation situated in exon 11 and the other is non-coding located in intron 9/10. For detailed information about the polymorphisms, see Table 3**.** Translating the sequence in exon 11 shows that the substitution changes a C nucleotide in the wild type to a T in the Silver allele. This will change the amino acid from an arginine to a cysteine.

Association of DNA polymorphism and the silver phenotype
To investigate the nucleotide substitution silver horses as well as non-silvers in different breeds were tested for the SNP using pyrosequencing. In total 25 silver horses from three breeds and 55 non-silvers of different colours from 11 breeds were successfully genotyped for the SNP. For detailed information about the number of individuals from each breed, see Table 4. Individuals with an ambiguous result of the genotyping was re-typed or sequenced.

All tested silver dappled horses had the genotype T/C (silver heterozygote) or T/T (silver homozygote), while the non-silvers all had C/C. This means a complete association between this polymorphism and the silver phenotype.

Discussion

TKY284 is situated near the *SILV* gene at chromosome 6, and therefore this result gives a support for *SILV* being the causative gene for the phenotype. The other markers that show linkages are situated close to each other and show that the linkage analysis is accurate. The DNA-sequence for *SILV* differed from the one already published in exon 6. These differences in DNA-sequence also lead to differences in the amino acid sequence. When aligning the two different amino acid sequences to other SILV-homologues, the sequence obtained in this project aligns more well than the sequence already published. Because of the large number of animals sequenced in this project, the distinct sequences and the alignment with the protein in other species, the sequence obtained in this project seems correct.

In the part of the *SILV*-gene that was sequenced, two polymorphisms showing linkage to the silver phenotype were found. One of these was a mutation in the intron, while the third one is a missense mutation in the last exon. As mentioned above, this mutation changes the fifth base pair in the last exon from cytosine to a thymine. In turn, this will change the second amino acid in the cytoplasmic region from an arginine that is positively charged, to a cysteine, that is a non-polar sulphur-containing amino acid. To investigate this mutation further, silver and non-silver individuals from different breeds were genotyped with pyrosequencing. This mutation showed a complete association with the phenotype in this material, i.e. all silver heterozygotes were C/T, silver homozygotes T/T while the wild-type horses had a C/C.

To know for sure what mutation that is responsible for the silver phenotype it is necessary to sequence the rest of the gene. In many of the other species that carry mutations in the SILV/Pmel17 several SNPs, insertions and deletions are found. But there is a possibility that the introduced Cys is enough to disrupt the protein domain in the beginning of the cytoplasmic region. This region of the Pmel protein is a rather well conserved region between species. Of the mammals, the majority has at least two arginines in the beginning of the cytoplasmic region. Also the chicken and other vertebrates have arginines in these positions. For more detailed information about the amino acid sequence among the vertebrates, see Table 5. The missense mutation in the cytoplasmic region is interesting, not only because of the conserved region, but also because the very same mutation has been found in Pmel17 of chicken. This mutation in chicken is associated with the dun phenotype. In the dun phenotype however, more mutations were found such as a deletion of five amino acids in the transmembrane region. It is still not known what mutation that causes the phenotype in chicken, which like the silver phenotype in horse leads to a diluting effect on the black pigment (Kerje et al., 2004). But our findings so far argue for that the identified missense mutation is the one responsible for the silver phenotype.

More silver and non-silver horses from different breeds will be tested for the mutation in the beginning of the cytoplasmic region using pyrosequencing. To investigate it further, the mutation will be introduced into a DNA-construct of Pmel that then can be transfected into mouse or human cell lines. By this, the function of Pmel and the function of the mutation can

be further studied. It would also be interesting to see if the silver dappled colour is associated with less number of melanocytes or less melanin. This could be done by taking biopsies from silver and non-silver horses that then can be investigated by a dermatologist.

As mentioned before, there are still many pigmentary disorders for which the genetic background is unknown (Passeron et al., 2005). Of the human pigment genes that have been cloned, almost all are associated with hereditary pigment diseases (Kushimoto et al, 2001). Identifying genes and mutations for a pigmentary disease or a pigment phenotype can increase the understanding of the function of the protein and the molecular mechanisms behind the process of pigmentation and pigmentary changes. By understanding these mechanisms there can be new ways to treat pigmentary disorders (Passeron, 2005). This makes identification and characterisation of the genes behind pigmentation even more interesting and important.

Acknowledgements

First of all I would like to thank Gabriella Lindgren for her excellent guidance through this project. Your support and help meant much! Thank you also to Sofia Mikko for giving me the opportunity to take part in the project. To all members of the lab, both at BMC and Ultuna, thank you for the help with DNA-preparation, sequencing and answering my endless questions!

References

Alberts, B. (2002). Molecular biology of the cell. Garland science, New York, USA.

Bailin, T., Lee, S.T. and Sprirtz, R.A. (1996). Genomic organization and sequence of D12S53E (Pmel 17), the human homologue of the mouse silver (si) locus. J Invest Dermatol 106: 24-27.

Basu, J. (2004). Protein palmitoylation and dynamic modulation of protein function. Current science 87: 212-217.

Berson, J.F., Harper, D., Tenza, D., Raposo, G. And Marks, M.S. (2001). Pmel17 initiates premelanosome morphogenesis within multivesicular bodies. Mol. Biol. Cell. 12: 3451-3464.

Bowling, A.T.(1996). Horse genetics. University Press, Cambridge, UK.

Brown, T.A. (2002). Genomes. BIOS scientific publishers, Oxford, UK.

Bycroft, M., Bateman, A., Clarke, J., Hamill, S.J., Sandford, R., Thomas, R.L. and Chotia, C. (1999). The structure of a PKD domain from polycystin-1 - implications for polycystic kidney disease. EMBO J. 18: 297-305.

Clark, L.A., Wahl, J.M., Rees, C.A. and Murphy, K.E. (2006). Retrotransposon insertion in SILV is responsible for merle patterning of the domestic dog. Proc Natl Acad Sci. (Epub ahead of print).

Dunn, L.C. and Thigpen, L.W. (1930). The silver mouse: a recessive color variation. J. Heredity 21: 495-498.

Ellegren, H. (2004). Microsatellites: Simple sequences with complex evolution. Nature reviews Genetics 5: 435-446

Furugren, B. (2000). Hästens färger. Natur och kultur, Stockholm, Sweden.

Green, P., Falls, K. and Crooks, S. (1990). Documentation for CRI-MAP, Version 2.4. Washington university School of Medicine, St Louis, MO.

Keeling, L., Andersson, L., Schutz, K.E., Kerje, S., Fredriksson, R., Carlborg O., Cornwallis, C.K., Pizzari, T. And Jensen, P. (2004). Feather-pecking and victim pigmentation. Nature 431, 645-646.

Kerje, S., Sharma, P., Gunnarsson, U., Kim, H., Bagchi, S., Fredrison, R., Schutz, K., Jensen, P., von Heijne, G., Okimoto, R. And Andersson L. (2004). The Dominant white, Dun and Smoky color variants in chicken are associated with insertion deletion polymorphisms in the PMEL17 gene. Genetics 168: 1507-1518.

Kim, R.Y. and Wistow, G.J. (1992). The cDNA RPE1 and monoclonal antibod HMB-50 define gene products preferentially expressed in retinal pigment epithelium. Exp. Eye. Res. 55:657-662.

Kobyashi, T., Urabe, K., Orlow, S.J, Higashi, K., Imokawa, G., Kwon, B.S., Potterf, B. And Hearing, V.J. (1994). The Pmel 17/silver locus protein. Characterization and investigation of its melanogenic function. J. Biol. Chem. 269: 29198-29205.

Kushimoto, t., Basrur, V., Valencia, J., Matsunaga, J.m Vieira, W.D., Ferrans, V.J., Muller, J., Appella, E. and Hearing, V.J. (2001). A model for melanosome biogenesis based on the purification and analysis of early melanosome biogenesis based on the purification and analyss of early melanosomes. Proc. Natl. Acad. Sci. USA. 98: 10698-10703.

Kwon, B.S., Chintamaneni, C., Kozak, C.A., Copeland, N.G., Gilvert, D.J., Jenkins, N., Barton, D., Francke, U., Kobayashi, Y. And Kim, K.K. (1991). A melanocyte-specific gene, Pmel 17, maps near the silver coat color locus on mose chromosome 10 and is in a syntenic region on human chromosome 12. Proc. Natl. Acad. Sci. USA. 88: 9228-9232.

Liu, B.H. (1998). Statistical genomics: linkage, mapping and QTL-analysis. CRC Press, Boca Raton.

Maresh, G.A., Marken, J.S., Neubauer, M., Aruffo, A., Hellström, I., Hellström, K.E. and Marquardt, H. (1994). Cloning and expression of the gene for the melanosoma-associated ME20 antigen. DNA Cell. Biol. 13: 87-95.

Marklund, S. (1997). Applied molecular genetics in domestic animals with particular focus on the horse. Dissertation Uppsala: Swedish university of agrigultural sciences.

Martinez-Esparza, M., Jiménez-Cervates, C., Bemmett, D.C. Lozano, J.A., Solano, F. And García-Borrón, J.C. (1999). The mouse *silver* locus encodes a single transcript truncated by the *silver* mutation. Mamm. Genome 10: 1168-1171.

Nichols, S.E., Harper, D.C., Berson, J.F. and Marks, M.S. (2003). A novel splice variant of Pmel17 expressed by human melanocytes and melanoma cells lacking some of he internal repeats. J. Invest. Dermatol. 121: 821-830.

Passeron, T., Mantoux, F. And Ortonne, J-P. (2005). Genetic disorders of pigmentation. Clinics in Dermatology 23: 56-67.

Rieder, S., Stricker, C. Joerg, H., Dummer, R. And Stranzinger, G. (2000). A comparative genetic approach for the investigation of ageing grey horse melanoma. J. Anim. Breed. Genet. 117: 73-82.

Ronaghi, M., Uhlen, M. and Nyren, P. (1998). A sequencing method based on real-time pyrophosphate. Science 281, 363-365.

Slominski, A., Tobin, D.J., Shibahara, S. and Wortsman, J. (2004). Melanin Pigmentation in Mammalian Skin and Its Hormonal Regulation. Physiol Rev. 84: 1155-1228.

Sponenberg, P. (2003). Equine color genetics. 2nd ed. Iowa state press, Iowa, USA.

Theos, A.C., Truschel, S.T., Raposo, G., Marks, M.S. (2005). The Silver locus product Pmel17/gp100/Silv/ME20: controversial in name and in function. Pigment Cell. Res. 18: 322-336

Picture 1. A silver black icelandic stallion.
Photo: Tim Kvick

Picture 2. A silver brown icelandic mare.
Photo: Tim Kvick

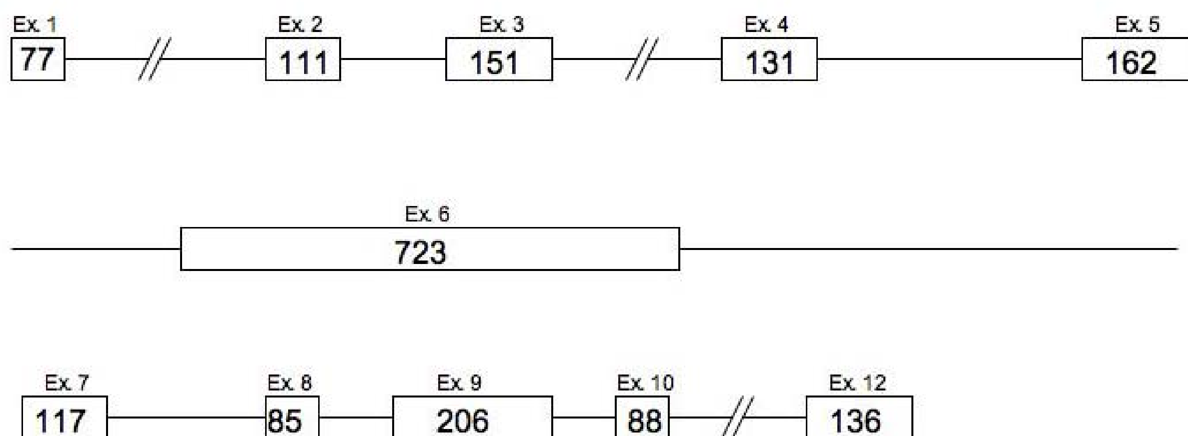

Figure 1. Human *SILV* gene with exon/intron organization and exon length.

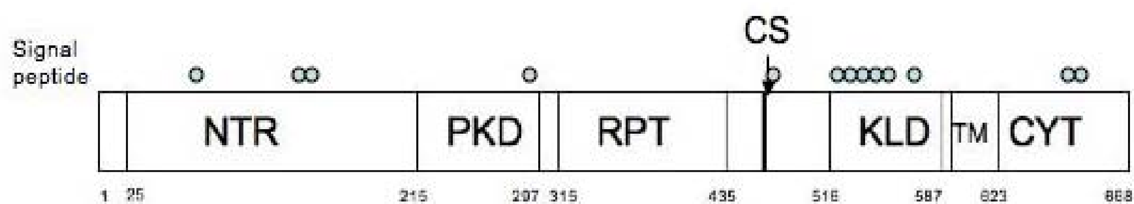

Figure 2. Domains and cystein residues (o) in the long form of human Pmel17. Reference: Theos et al., 2005

.

Table 1. Microsatellite chromosomal locations, annealing temperatures and stallion genotype (OO for homozygous and AB for heterozygous).

ECA	Marker	Annealing temp.	Stallion genotype
1	VIAS-H45	58	OO
1	LEX020	58	AB
1	ASB41	58	OO
1	NVHEQ100	58	AB
1	COR100	56	OO
1	ASB8	58	OO
2	ASB18	58	OO
2	COR065	58	AB
2	A14	58	OO
3	COR033	58	AB
4	COR089	58	AB
5	LEX034	58	AB
5	LEX004	58	OO
6	COR070	58	OO
6	TKY284	57	AB
6	NVHEQ82	58	OO
8	LEX023	58	AB
8	COR056	69	OO
8	COR003	58	OO
9	COR008	58	AB
9	UM037	58	AB
10	NVHEQ18	58	OO
10	ASB9	58	OO
12	COR058	58	OO
13	COR069	58	OO
13	VHL047	58	AB
14	COR002	58	OO
15	B-8	58	AB
15	COR075	58	AB
16	LEX056	58	OO
16	I-18	58	OO
17	NVHEQ79	58	AB
18	LEX054	58	OO
19	LEX073	58	AB
20	LEX052	58	AB
20	UM011	58	OO
21	COR073	58	AB
21	LEX037	56	OO
22	HMS47	58	OO
22	HTG21	58	AB
23	COR055	58	AB
24	COR061	58	AB
24	COR024	58	OO
26	A-17	58	AB
26	COR071	58	OO
27	COR040	58	OO
27	COR031	58	AB
29	L12.2	58	OO
30	LEX025	58	OO
X	LEX022	58	OO

Table 2. Primer sequences for sequencing of *Equus caballus* SILV an pyrosequencing primers

Primer	Forward primer (5'- 3')	Reverse primer (5' – 3')
Exon 5-6	TGCCCCTCGCTCACTCCCGCTCAGCCT	CATGAATGGGCTGGCATCTGGA
Exon 6-7	GGTAACGGTACAGAGTTGGTGGAA	GGACGATGTCCAGAGTGAGGGA
Exon 6-7(2)	AGGTGCCAACTGCAGAGC	GGACGATGTCCAGAGTGAGG
Exon 7-Intron 8	ATGGCACAGCCACCTTATTC	GAAAGGTGTCAGTTTAGGTCAG G
Exon 7-10	CCAGAGCCCCCTGCTGGATGG	TATATCAGAGATGCAAGCACCA TA
Exon 9-Intron 10	AATGTGTCTTTGGCTGATGC	TCTGCCCCTCTTACAGGTGA
Intron 10-Exon 11	GCAGGGAAGCTTGTAGAGTGA	CTCTCACCAAAGGGGGAAG
Exon 10-11	AGAGGCAGGCCTTGGGCAG	TGCTCTCACCAAAGGGGGAAG
Pyroseq. PCR primers	TCCATTGCTTACCAGTTTCCTT	CTCACCAAAGGGGGAAGAG
Pyroseq. Sequencing primer	-	GCCCTGCTTCATAAGTCTG

Table 3. Polymorphisms associated with the silver phenotype

Colour	Intron 9 bp 48	Exon 11, bp 5
Non-silver	A/A	C/C
Silver heterozygote	A/T	T/C
Silver homozygote	T/T	T/T

Table 4. Number of individuals from each breed genotyped for the SNP in exon 11

Breed	Silver horses	Non-silver horses
Icelandic horse	22	33
Ardenne	1	-
Swedish warmblood	2	-
North Swedish horse	-	3
Shetland pony	-	3
Welsh pony	-	3
Norweigian fjord horse	-	2
Connemara pony	-	2
Thoroughbread	-	3
Haflinger	-	4
New Forest pony	-	2
Total	**25**	**55**

Table 5 Amino acid sequence in the beginning of the cytoplasmic region *SILV*-homologues

Species	**Amino acid sequence**									
Equus caballus	R	R	-	-	-	-	R	L	M	K
Equus caballus (silver)	R	C	-	-	-	-	R	L	M	K
Homo sapiens	R	R	-	-	-	-	R	L	M	K
Mus musculus	R	H	-	-	-	-	R	L	K	K
Canis familiaris	R	R								
Rattus norvegicus	R	R	-	-	-	-	R	L	K	K
Bos taurus	R	R	-	-	-	-	R	L	M	K
Gallus gallus	R	R	V	K	Y	S	P	L	L	P
Gallus gallus (dun)	R	C	V	K	Y	S	P	L	L	P