Swedish University of Agricultural Sciences

Department of Animal Breeding and Genetics

Genetic Characterization and Inheritance of Belly Spot and Splashed White Coat Color in Horses

by

Helena Eken

Supervisor:

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Examensarbete 307

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Agrovoc: Pigmentation, horses, microsatellites, markers, chromosome mapping, genetic markers
Övrigt: Coat color

Supervisor: *Sofia Mikko, HGEN, SLU*

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Abstract

Coat color studies are important because in some breeds only certain colors are permitted; another reason is that some coat colors are associated with diseases. The purpose of this master thesis is to shed some light on the inheritance pattern of the splashed white coat color and belly spot in horses. There is so far no indication that splashed white or belly spot would be associated with a disorder. The Swedish Warmblood (SWB) was chosen for the belly spot phenotype and the Icelandic horse was chosen because they show the splashed white phenotype. One hypothesis is that there is a similar genetic background to these phenotypes.
For the study half sib families to six SWB stallions and one Icelandic horse stallion have been used. The samples for the study were collected from the Animal Genetics Laboratory at the Swedish University of Agricultural Science. The material includes 356 horses in total; they have been genotyped for 8 markers close to three candidate genes. Genotypes were determined by PCR amplification of markers and capillary electrophoresis. Pedigree information and phenotypic information have been used to perform an association study and linkage analysis on the results. The phenotypic and pedigree information was retrieved from the SWB Association (ASVH). The inheritance study of the half-sib families included all offspring and mares to each of the SWB stallions.
Splashed white, belly spot and white leg markings show a complex inheritance pattern. The *Kitligand* gene is most likely involved in the splashed white and belly spot phenotypes while the *Kit* gene is likely to be involved in the white leg markings. White facial markings are most likely controlled by one major gene *Kit*, blaze seem to be a dominant trait whereas white lower lip seem to be recessive. The extension of white markings is affected by the coat color; chestnuts have more extensive white pattern while black and bay horses have more restricted areas of white.

Sammanfattning

Studier på pälsfärger är viktigt, i vissa raser är det enbart ett fåtal godkända färger, det finns även exempel på färger som är associerade till olika sjukdomar. Studier kring färgernas genetiska bakgrund kan därmed underlätta fortsatt forskning. Syftet med detta arbete var att få en inblick i nedärvningen för bukskäck och bukfläck på häst. Det finns inga misstankar om att dessa färger skulle vara kopplade till sjukdomar. Svensk varmblodig ridhäst (SWB) valdes för att studera bukfläcksfenotypen och islandshästen för att studera bukskäck. En av hypoteserna inför arbetet var att det finns en gemensam genetisk bakgrund till dessa två fenotyper.
I studien användes familjematerial från sex SWB hingstar samt en islandshäst hingst. Proverna samlades från det husdjursgenetiska laboratoriet på Sveriges Lantbruksuniversitet. Materialet inkluderade totalt 356 hästar; dessa typades för åtta genetiska markörer (mikrosatelliter) nära kandidatgenerna. De genotypades genom PCR amplifiering och kapillär elektrofores. Till genotypinformationen lades härstamnings- och fenotypinformation för att utföra associations- och kopplingsanalyser. Härstamnings- och fenotypinformationen hämtades från Avelsföreningen för Svenska Varmblodiga Hästen (ASVH). För att studera nedärvningen användes halvsyskonmaterialet för SWB hingstarna, som kontroll grupp användes stona.
Bukskäck, bukfläck samt vita tecken på benen visar en komplex nedärvning. *Kitligand* genen är troligen involverad i bukskäck och bukfläcksfenotyperna medan *Kit* sannolikt är inblandad

i vita tecken på benen. Vita tecken i ansiktet styrs förmodligen av en huvudsaklig gen, *Kit*. Utbredningen av vita tecken påverkas av grundfärgen; fuxar har mer utbredda vita tecken medan svarta och bruna hästar har mer begränsade vita tecken.

Introduction

Why coat color studies?

There are several reasons for studying the coat color in horses. In many horse breeds only some colors are allowed if the horse should be registered. These requirements put pressure on the breeders to produce horses that have a certain color or at least do not have one of the unaccepted colors. This practice is common within dog and cat breeding as well. A further reason is that the melanocytes, which produce pigment, originate from the neural crest as the neurocytes. They also share the same paths during their migration in the body. Some coat colors are associated with neurological and other disorders, and could therefore be of interest, for example frame overo coat color which is associated with the Overo Lethal White Syndrome (OLWS).

Purposes and hypothesis

The purpose of this study is to investigate the inheritance pattern of splashed white and belly spot. Another purpose is to find a pattern between more or less extensive white markings.

Hypothesis 1.
- The genes involved in splashed white coat color are the same as in belly spot. There are several genes involved in both traits.

Hypothesis 2.
- Swedish Warmblood (SWB) horses that have extensive white markings are heterozygous while horses with extensive white markings, belly spot and possibly blue eyes are homozygous.
- In Icelandic horses the individuals with blaze and occasionally white leg markings are heterozygote while splashed white horses are homozygous

The blue eye phenotype had been recorded so few times in this material that this trait was not further investigated.

Hypothesis 3.
- Chestnut colored horses have more extensive white markings.

The biology behind coat colors

The melanocyte

The melanocyte and neurocytes share origin from the neural crest; from there they spread through the body during the embryonic development. Melanocytes are the pigment producing cells in the body. The neurocytes constitute the nervous system. The migration from the neural crest is finalized by the colonization of the hair follicles during fetal stage. It is not until postnatal that the cells differentiate into melanocytes and start the production of pigment. This emigration is referred to as the melanogenesis and several factors affect this migration, among them Endothelin receptor β (Ednrb) and a receptor tyrosine kinase (Kit) (Kissel, H. et al, 2000).
If the melanoblasts are inhibited during the migration to the fur/skin that area will lack pigment cells and will therefore appear white. The pigments produced by the melanosomes absorb light of certain wavelength which is visualized as a color of the fur/skin.

Kit and the *Kitligand*

In the cell wall of the melanocyte several proteins are attached, among them Kit, a transmembrane tyrosine kinase receptor. Kit has a ligand called Kitligand, steel or stem cell growth factor (SCF) (Alberts B. et al, 2002). From previous studies we know that Kit is involved in fetal development. The *Kit* gene is only expressed in melanocytes of the hair, skin and choroids (Aoki H. et al, 2005). Mutations in the *Kit* gene or the *Kitligand* gene in human and mouse can cause anemia, sterility and white spotting in the skin. The Kitligand is produced by the surrounding cells during migration and also at the settle point for the melanocyte. It is important that both Kit and the Kitligand are functional for the migration and survival of the melanocytes (Alberts B. et al, 2002)
Lahav (2005) found in his study that a mutation in the c-*Kit* gene in mice and humans causes "absence of pigmentation from forehead, eyebrows and chin and of the ventral chest, abdomen and extremities". Aoki et al (2005) showed in their study that homozygote's for a mutation in the *Kit* gene have a band across the body. They also showed that the homozygotes in some cases could compensate the mutation in the *Kit* gene by the *Edn3* gene, these individuals showed spots in the trunk region and white markings in the face. They also suggest that the expression of the *Kit* genotype is dependent on the *Edn3* genotype, which in some cases could compensate quite much of the lack of signaling in the *Kit* gene.

Pax3

Pax3 is a transcription factor that is essential for the development of melanin producing cells in vertebrates. It is early expressed in the embryo and plays an important role in the regulation of melanocyte lineage. The *Pax3* gene is also reported to be encoded again in melanoma, skin cancer. The temporal expression of the *Pax3* gene is linked to the melanoblast proliferation or migration during melanogenesis (Blake, J. and Ziman, M. 2005).
Pax3 activates the gene by attracting other transcription factors. There are alternate isoforms within the Pax3 family, and these isoforms exhibit altered transcriptional activity and specificity. The different isoforms end up in different end stages, the ones that travel to the hair follicles are Pax3d-positive cells. It is known that Pax3 is involved in the regulation of melanin synthesis by regulating genes such as Mitf and tyrosine-related protein-1. Pax3 can

however also silence these to keep them undifferentiated in mature skin. It is although believed that Pax3 regulate proliferation and/or migration to a greater extent than quiescence. Pax3 has a conserved role in early development of the melanoblasts prior to differentiation and melanin production (Blake, J. and Ziman, M. 2005).

Coat colors

Splashed white

This coat color has been classified by Sponenberg (1996) in the same group as frame overo and sabino. Frame overo and splashed white horses are easily separated and have distinct differences in their patterns. The splashed white coat color is according to Sponenberg (1996) difficult to distinguish from the sabino coat color. A splashed white colored horse looks as if it has been dipped in white paint; the legs are white and to varying extents also the ventral part of the body. Splashed white colored horses usually have a dominantly white head with blue eyes. The background color could be black, brown or chestnut and the extension of the white fields differs from individual to individual. The inheritance is believed by some to be of dominant nature but is very rare and easily confused with sabino. The splashed white is considered to be a variant of the wider group of overo colored horses. According to Sponenberg (1996) homozygotes have not been recorded. In Iceland the splashed white coat color is believed to be recessive and only appears when the horse is homozygous as the horses in figure 1a. Heterozygous horses are believed to sometimes have a white blaze and blue eyes (see figure 1b.).

a.

b.

Figure 1. Splashed White in the Icelandic horses. (a) possible homozygote's and (b) possible heterozygote. (Photos by Tim Kvick)

Sabino

Sabino is, as mentioned earlier, a very similar coat color to splashed white color and it is sometimes difficult to tell the difference. According to Sponenberg (1996) most splashed white horses in North America are recorded as sabino. This makes it difficult to determine the inheritance pattern of the splashed white as well as the sabino. He also classifies sabino as an under group to overo. It is shown as high white markings on the legs, always complemented by facial white. In contrast with the two other described patterns, sabino is more widely spread between breeds. Sabino coat color is characterized by crisp clean

markings and may be confused with frame overo. Frame overo horses rarely have white legs, sabino horses have white legs. A minimally colored sabino completely lack white body markings and may only have white socks and facial markings. The minimally marked horses are rarely classified as spotted but can produce spotted offspring. This provides another complexity in determination of inheritance pattern. (Sponenberg, D. P, 1996)
Brooks and Bailey (2005) have studied the genetics behind the sabino1 (SB1) spotting pattern and have revealed that an exon skipping in the *Kit* gene is responsible for the coloring. The exon skipping is due to a point mutation in intron 16, and exon 17 is subsequently removed. Complete linkage was found between the SNP in intron 16 and the SB1 phenotype in the Tennessee Walking horse families that were tested. The horses with sabino color could still produce the correct RNAstrand but most lack exon 17. The extensiveness of the white color differed largely between the individuals probably due to a combination of other spotting/coloring patterns. The homozygote's for the mutation were almost completely white (Brooks, S. A. and Bailey, E., 1996). In their study Brooks and Bailey determined the phenotypic appearance of a horse with sabino 1 markings as having at least three of the following four characteristics: (1) two or more white feet or legs, (2) blaze, (3) jagged margins around white areas and (4) spots or roaning in the midsection.

Tobiano

Tobiano is in a way the opposite of splashed white; tobiano horses have the same base colors but the white covers the dorsal part of the body, white feet and lower legs is common. Within the white fields small spots of color so called ink spots can appear, common among homozygotes. Tobiano markings have clean edges (Sponenberg, D. P., 1996).
Tobiano markings have in a study by Brooks et al (2002) shown to be associated with the *Kit*-gene. In their study the coat color markings showed a clear connection with a polymorphism in intron 13 of the *Kit*-gene. 129 unrelated tobiano colored horses were compared to 104 solid colored thoroughbred horses. All tobiano horses were either heterozygous or homozygous for the mutation at intron 13 in the *Kit*-gene; whereas, only three of the solid colored thoroughbred horses were heterozygous for the same change. The *Kit* gene is therefore believed to be responsible for the tobiano coat color (Brooks, S. A. et al, 2002). The causative mutation is not known.

Colors and white markings within the Swedish Warmblood

SWB are mainly solid colored horses, with occasional white markings on head and legs. There are no notations regarding coat colors in the breeding goals (http://www.asvh.se). Little is known about white markings in horses although literature on mice and humans indicate that the extension of white areas can vary to a great extent even within the same genotypes. Some SWB's however can have a white belly spot, see figure 2a. The white belly spot is believed to be associated with extensive white markings on the rest of the body, see figure 2b. The main characters are a wide blaze, several legs with white markings often also a white lower lip. It has also been found that blue eyes are a common trait, probably due to the wide blazes. Further on it is well known that chestnuts have more extensive white markings compared to bay or black horses, see figure 2.

a.

b.

Figure 2.
a. A chestnut colored foal with extensive white markings and a large belly spot. b. A bay mare with typical white markings associates with belly spot. (Photos Ann-Charlotte Mellden)

Colors and white markings within the Icelandic horses

Icelandic horses show a great variation of colors and most colors are accepted for registration. In the Icelandic horse breeding goals it is written that the great color variation should be preserved (http://www.feif.org/). In contrast to the SWB, white markings on the legs are rare. Blaze and occasionally a white lower lip are more common. In Iceland the splashed white horses are believed to be homozygous while the horses with just a blaze and occasionally white markings on the legs are heterozygous.

Materials and methods

Family material

For this study, half sib families from six SWB stallions and one Icelandic horse stallion were used. Genotypes were determined for the seven stallions, 222 offspring and 73 mares.
The SWB was chosen because the breed has a low variability in coat color; solid colored horses are preferred, although all colors are accepted. White markings are more common within the breed than paint colored horses. The six stallions were selected for this study because they give a large proportion of offspring with extensive white markings on face and leg; some even give offspring with white belly spots. These horses are not tobiano or frame overo since these colors are not extensively spread in this breed. This facilitated the determination of the origin of extensive white marking in the offspring.
The records on the SWB are good in Sweden although some traits are more commonly not noted like belly spots and blue eyes. The belly spot is one of the most interesting traits in this study and there is a possible similarity between the belly spots and the splashed white. The rare notations of belly spots and blue eyes have complicated this work.
The six SWB stallions used for the study showed a range of phenotypes; chestnut, bay and black base colors were all included. White face markings included a thin blaze to baldface, and they had between three and four white stockings. Four of the stallions had a belly spot and two were slightly roaned.

The Icelandic stallion was chosen because the stallion himself is not splashed white but about half of his offspring show extensive white markings and a few are splashed white. The stallion himself is chestnut with only a blaze and no other white markings.

The candidate genes

Kit and the *Kitligand* gene were chosen for their role in development and proliferation of the melanocytes. *Kit* gene is known to be involved in white spotting in other animals and is therefore a highly interesting candidate gene for the splashed white coat color.
Pax3 gene has been chosen as a candidate gene for splashed white since it is a transcription factor and is therefore involved in gene expression regulation and this trait has a high variance in expression.

Mode of inheritance

The phenotypes of all offspring to the selected SWB stallions were collected from the Swedish Warmblood Association (ASVH). The phenotypes were used in an analysis to clarify the mode of inheritance for white markings. Phenotype recordings also gave more information regarding phenotypes of the horses included in the genetic study. Phenotypes of all the horses included in the study were noted. The dams of the offspring to the selected stallions were used as a control group for the inheritance study, which included 1346 offspring to the six SWB stallions and the control group contained 919 mares. The phenotypes retrieved from ASVH were also used for association and linkage analysis.

DNA samples

Hair, blood and pure DNA samples have been used. The hair and blood samples were prepared during the project while the pure DNA samples had previously been prepared for parentage testing. Most of the hair and blood samples were already available in the Animal Genetics Laboratory, Swedish University of Agricultural Science, were they had been used for parentage testing and identification. Some hair samples were previously collected, purely for the purpose of coat color studies. An ethical approval had been given from the Swedish Board of Agriculture for hair and blood sampling.
DNA from the hair was prepared by cutting of the follicles and treated with 0.18 µg/µl proteinase K, 1X PCR GOLD Buffer (without $MgCl_2$) (Applied Biosystems) and 1 mM $CaCl_2$ in 45% Tween 20 and 45% NP40 at 56 ^{o}C for two hours, after which inactivation of the Proteinase K was made at 95 ^{o}C for ten minutes.
Blood samples were prepared using blood DNA mini kit from Omega bio-tek Inc.
Microsatellites for the different candidate genes and their positions on the chromosome can be found in table 1.

Table 1. The markers that are surrounding the candidate genes. The number of alleles refers to the number of alleles found in this study.

			Sequence			
Locus	ECA	Reference	Forward	Reverse	Linked to candidate gene	Position
TKY353	3	Tozaki T. et al (2001)	TGTCACTGACA GATGAATGG	TGCCACCACTG ACAACAAAC	*Kit*	41.8 Mbp
LEX057	3	Coogle L and Bailey E. (1997)	TGGTCCCCTAA TCAAATCAGA	ACGGCATCCCA CATAAAATAG	*Kit*	64.0 Mbp
ASB23	3	Lindgren, G. et al (1998)	GAGGTTTGTAA TTGGAATG	GAGAAGTCATT TTTAACACCT	*Kit*	72.7 Mbp
HTG30	28	Lindgren, G. (2000)	TCAAGGCAAAT CTTTCCCAG	GTAAAATAACA AGTTGTTCCAG	*Kitligand*	13.4 Mbp
TKY319	28	Tozaki T. et al (2000)	TATGCACGAGA TTAAACGGG	AAAGAAGTCAG ATGAGCAGG	*Kitligand*	25.4 Mbp
COR10	6	Hopman, T.J. et al (1999)	TTGAAGGGTGG AGTAGGG	GACAAGAAGG GATGAAGGAG	*Pax3*	7.2 Mbp
NVHEQ82	6	Bjornstad, G. et al (2000)	TGTGGCAGCAT CCCACAAAC	CCTCCATTTTTG TCGGTTAGCG	*Pax3*	15.6 Mbp
TKY 1001	6	Tozaki, T. et al (2004)	TCTCAGAAGCC ATCTGGAG	ATCGATGCAGA ACACGTGG	*Pax3*	11.9 Mbp

Kit is positioned on ECA3 at 71.2 Mbp the closest located marker is ASB23 1.5 Mbp away and the marker positioned the furthest away is TKY353 29.4 Mbp away, see figure 3a. *Kitligand* is positioned on ECA28 at 14.6 Mbp. HTG30 is positioned only 1.2 Mbp away and TKY319 10.8 Mbp away, see figure 3b. *Pax3* is positioned on ECA6 at 11.4 Mbp. TKY1001 is positioned closest to the gene 0.5 Mbp away, COR10 and NVHEQ82 are both positioned 4.2 Mbp away but on opposite sides of the gene, see figure 3c.

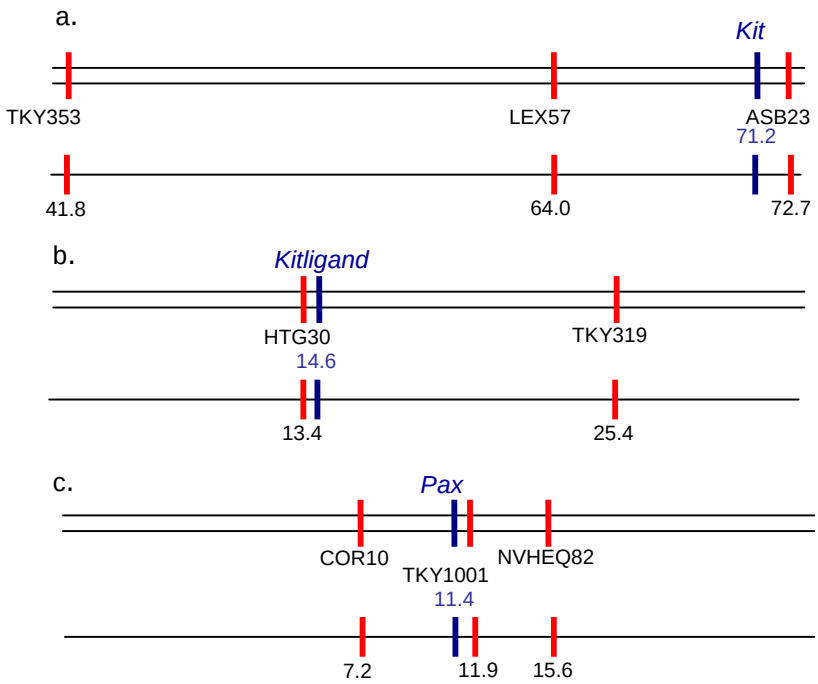

Figure 3. The positions of the markers and the candidate genes. Distances are in mega base pairs (Mbp). In picture a the part of ECA 3 where the *Kit* gene is positioned can be seen, in b the part of ECA 28 where *Kitligand* gene is positioned can be seen and in c the part of ECA 6 where *Pax3* gene is positioned can be seen.

The PCR

Prior to the PCR, the samples were diluted with ddH_2O. DNA preparation from hair was diluted 1:1, DNA prepared from blood by QiaAmp or OmegaKit 1:50 and pure DNA (approximately 0.5-1.0 ug/ul) samples 1:1000, regardless of initial concentration. Empirically it has been found that these dilutions work well for the PCR amplifications of the microsatellites. This dilutes the melanin which can inhibit the PCR reactions.

PCR reactions were performed using the standard constitution of 1X PCR GOLD Buffer (with $MgCl_2$) (Applied Biosystems), 0.2 µM dNTP, 0.05 U/µl AmpliTaq Gold. For LEX57, HTG30, TKY353 and COR10 0.1µM forward primer, 0.5 µM reverse primer and 0.5 µM M13-linker was used. For TKY319 and TKY18 0.33 µM of each primer was used. For ASB23 0.5 µM of each primer was used. For NVHEQ82 0.1 µM of each primer was used. For TKY1001 0.67 µM of each primer was used. In each reaction 1.0 µl of DNA (of various concentrations) was used. This amount had empirically proved to give good results. For the markers where M13-linker was used the linker had been labelled with fluorescent dye FAM, VIC, NED and PET. The markers were M13-linker was not used the forward primer had been labelled see table in appendix 1.

All individuals were genotyped for the 8 markers. The preparation of PCR followed a protocol from which the amount of primer and water was altered between markers. The protocol for each primer can be seen in the table in appendix 1. For the PCR programs the GeneAmp PCR System 9700 (Applied Biosystems) was used. The program protocols can be seen in appendix 2.

Also 155 individuals had previously been typed for the 17 markers included in the parentage testing kit (Equine Genotypes™ Panel 1.1, Finnzymes Diagnostics, Finland) these were also used as controls.

Capillary electrophoresis

The PCR products were analyzed by capillary electrophoresis using ABI PRISM™ 3100 Genetic Analyzer (Applied Biosystems). The PCR products were pooled together into groups determined by the size of the PCR fragments and the color they had been labeled with. 0.5 µl PCR product for each marker was added to each pool. To each well 0.25 µl Genescan 500 - LIZ™ Size Standard (Applied Biosystems) and 12 µl HiDi Formamide (Applied Biosystems) was added before starting the electrophoresis. After the addition of these, the mixes were denaturized for 3 minutes at 95 °C.

Table 2. The pools for the ABI PRISM™ 3100 Genetic Analyzer (Applied Biosystems) analysis.

	Markers
Pool 1	COR10, NVHEQ82, TKY1001
Pool 2	LEX57, HTG30, TKY353, ASB23, TKY319

The data from the ABI PRISM™ 3100 Genetic Analyzer (Applied Biosystems) were analyzed using the software program GeneMapper version 4.0. The results from GeneMapper were compiled into a table where all 8 markers and all 302 individuals were included. The results from GeneMapper analysis were later compiled with the phenotype data from ASVH and used in association and linkage analysis.

Association analysis

The association analysis was performed by calculating chi-square-values, using the software program Conting version 2.71 (Ott, J. 1988). All horses from the genetic analysis were ordered into groups according to their phenotypes and the alleles from each marker were counted. The stallions, mares and offspring were all included. Only one marker was analyzed at a time. Conting calculated chi-squares, degrees of freedom (d.f.) and p-values for the allele frequencies in the different groups. The groups are shown in Table 3.

Table 3. The classification of the different phenotypes used in the association analysis, the horses had a basic division depending on base color chestnut/not chestnut.

Face	**Description**
Blaze	White markings that cross the whole face but no white lower lip.
White lower lip	At least white spot on the lower lip, regardless of other markings.
Little white	White markings that does not stretch across the whole face.
No white markings	No white areas in the face.
Legs	**Description**
0 white legs	No white markings on any leg.
1 white leg	White markings on one leg.
2 white legs	White markings on two legs.
3 white legs	White markings on three legs.
4 white legs	White markings on four legs.
Belly spot	**Description**
Present	Any sort of white spot on the belly area.
Not present	No white areas on the belly area.

These groups were combined during the association analysis to see how the p-value was altered. This was made for all markers. For face the four groups was for a - same as described above with regards to color, b – as described above regardless of color, c – blaze and white lower lip versus little white and no white markings with regards to color and d - blaze and white lower lip versus little white and no white markings regardless of color. For the leg markings there were six different combinations a- according to the table above with regards to color, b – according to table above regardless of color, c – 0, 1-2 and 3-4 legs with white markings with regards to color, d – 0, 1-2 and 3-4 legs with white markings regardless of color, e – 0-1, 2-3 and 4 legs with white markings with regards to color and last f – 0-1, 2-3 and 4 legs with white markings regardless of color. Due to a limited number of horses with the belly spot phenotype they were all gathered into one group and compared to all others.
The Icelandic horses were too few to perform a complete analysis but results from the genotyping were analyzed manually and compared to the results found in the association analysis for the SWB.

Linkage analysis

The linkage analysis was performed using the software program Crimap (Green, P. et al, 1990). In the linkage analysis all markers were analyzed at the same time against the phenotypes. The phenotypes have to be determined as homozygous or heterozygous for the characters. The determination of genotypes behind the characters were determined based on the inheritance analysis and the association study and can be seen in Table 4. The linkage analysis analyzed white markings on face, on the legs and white belly spots.

Table 4. The classification of phenotypes for the linkage analysis expected genotypes in major gene.

Blaze	**Description**
Not present	Homozygous allele 1 or heterozygous allele 1/allele 2
Small	Heterozygous allele 1/allele 2
Wide	Homozygous allele 2
White lower lip	**Description**
Not present	Homozygous allele 1 or heterozygous allele 1/allele 2
Present	Homozygous allele 2
Leg markings	**Description**
0-1 white legs	Homozygous allele 1 or heterozygous allele 1/allele 2
2-3 white legs	Heterozygous allele 1/allele 2
4 White legs	Homozygous allele 2
Belly spot	**Description**
Present	Homozygous allele 1 or heterozygous allele 1/allele 2
Not present	Homozygous allele 2

Due to lack of time and too few horses in the Icelandic group there was no linkage analysis performed on these horses. The main difference between the association analysis and the linkage analysis is that the association analysis does not account for the relationship between the individuals. Another difference is that the association analysis does not need information regarding the expected genotype of the individuals based on their phenotypic appearance.

Results

Inheritance study

The results from the inheritance investigation are presented in Figures 4-6. The offspring of all the Warmblood stallions are gathered into one group and all the dams are in the other (control) group. The stallions are not included in this study and neither are the Icelandic horses due to that they are so few.

In Figure 4 it is shown that among the chestnut colored offspring it is tenfold more common with blaze than without. Among the chestnut mares it is twice as common to have a blaze. Among the non chestnut colored offspring it is twice as common to have blaze. Among the not chestnut colored mares it is only half as common to have blaze. In both these cases there is a strong significant difference between the offspring and control group, p-value < 0.001. In some cases the offspring do not have a blaze even though both parents have a blaze. There are cases when the dam does not have a blaze or any white in the face and the offspring has a blaze. Since all SWB stallions included in the study have at least blaze there are no examples of an offspring having blaze when neither of the parents do.

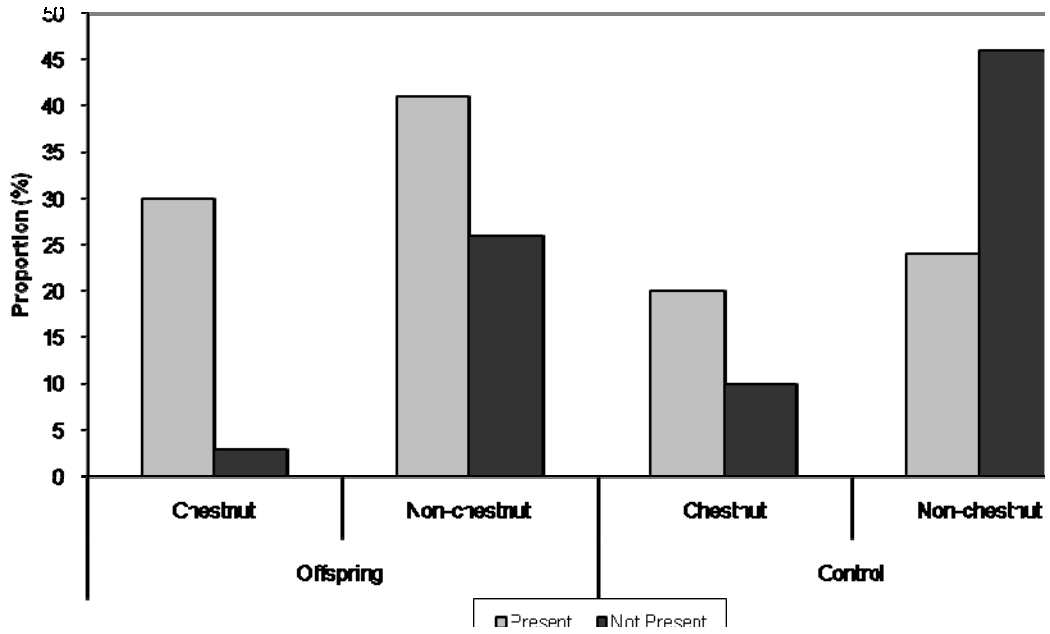

Figure 4. Frequencies of blaze in offspring and control group. Proportion meaning the proportion of horses within the two major groups (offspring and control) that show or do not show the phenotype.

Figure 5 show the same information regarding the white lower lip. Among the chestnut colored offspring twice as many have white lower lip. Among the chestnut mares half as many have white lower lip. Among the not chestnut colored offspring half as many have a white lower lip. Among the non chestnut colored mares only one sixth have white lower lip. There is a significant difference (p-value<0.001) both between control group and offspring and also between chestnut

and not chestnut regarding white lower lip. There are cases when neither of the parents have a white lower lip but the offspring have a white lower lip. There are also cases when both parents have a white lower lip and the offspring does not have a white lower lip.

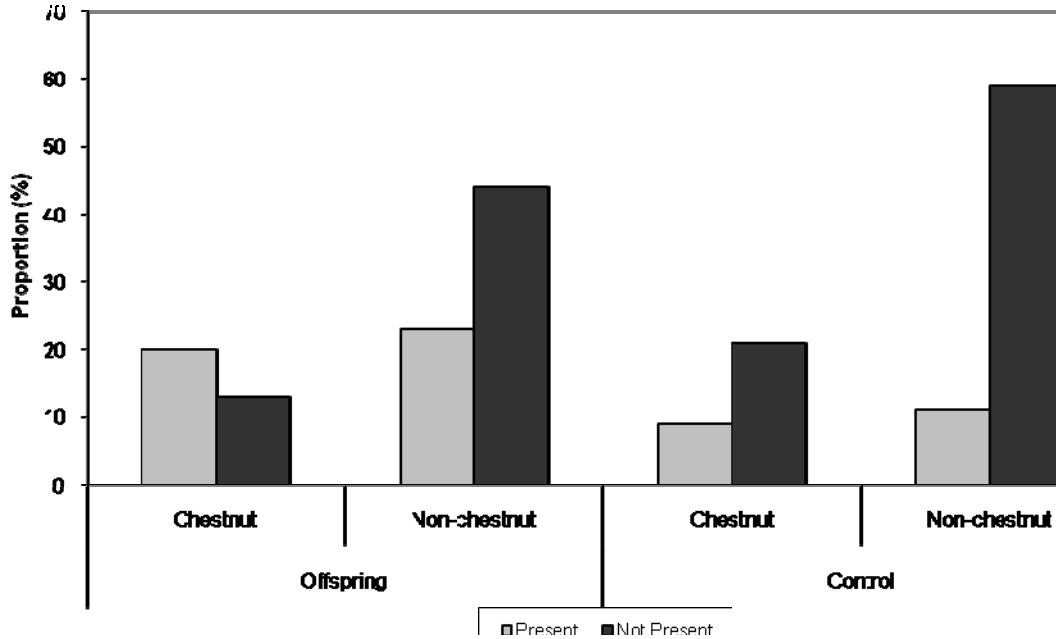

Figure 5. Frequencies of white lower lip in the offspring and control group. Proportion meaning the proportion of horses within the two major groups (offspring and control) that show or do not show the phenotype.

Figure 6 show the proportion of white leg markings among the offspring and the mares divided into chestnut and not chestnut. Among the chestnut colored offspring it is six times more common with four white leg markings than it is with no leg markings. Among the chestnut colored mares it is as common with four white leg markings as with no leg markings. There is high significance (p-value < 0.001) in the difference between the mares and offspring.
Among the non chestnut colored offspring it is three times more common to have four white leg markings than to have no white leg markings. Among the non chestnut colored mares it is half as common to have four white leg markings as to have no leg markings. There is a significant difference between the mares and offspring regarding four white leg markings and no white leg markings regardless of which color they are.

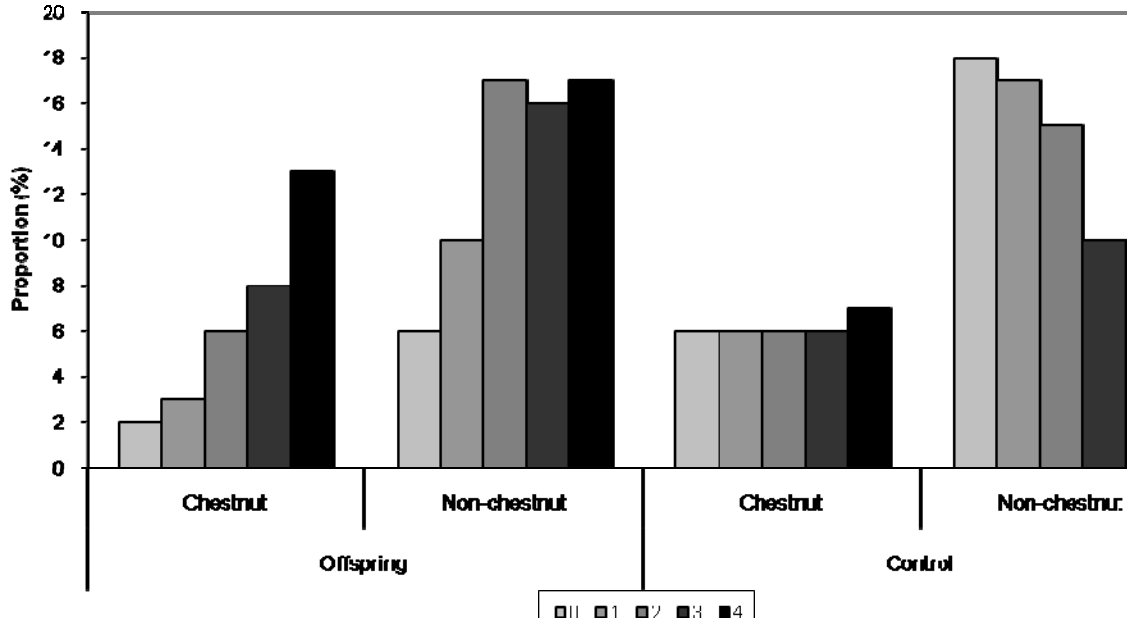

Figure 6. Frequencies of white markings on the legs in the offspring and control group. Proportion, meaning the proportion of horses within the two major groups (offspring and control) that show a certain phenotype.

Belly spots were almost equally frequent in the two groups; 3% in the offspring group and 2% in the control group.

Association study in SWB

The association study of the eight markers surrounding the three candidate genes showed significant results for all three groups of markings. To increase the certainty of the results three markers were used as control markers. These markers did not show any significant association.

Kit gene

The full table with the results from the Contig analysis for *Kit* gene for all traits is found in appendix 3. The most significant value for *Kit* gene on face markings were found for LEX57 when white lower lip, blaze, little white and no white were analyzed separately with regards to color, p-value <0.0001 which is highly significant. The highest p-value for *Kit* gene on face markings was LEX57 when white lower lip, blaze, little white and no white were analyzed separately regardless of color, p-value 0.51 which is not significant. Most p-values were between 0.01 and 0.001. All markers show significant values for the facial markings and several times the significance level is high (p-value<0.001).

The most significant value for *Kit* gene on leg markings were found for LEX57 when 0, 1, 2, 3 and 4 legs with white markings were analyzed separately with regards to color, p-value <0.0001 which is highly significant. The highest p-value for *Kit* gene on leg markings was LEX57 when 0-1, 2-3 and four legs with white markings regardless of color were analyzed, p-value 0.71 which is not significant. More than half of the p-values show more than one star significance (p-

value<0.05) and more than one third show a two star significance level (p-value<0.01). All *Kit* gene markers show significant association with leg markings.

The lowest p-value for *Kit* gene on belly spot was found for TKY353, p-value 0.13 which is not significant. The highest p-value for *Kit* gene on belly spot was ASB23, p-value 0.52 which is not significant either. Neither of the p-values for *Kit* gene markers shows significant association with belly spot.

Kitligand gene

The full table with the results from the Contig analysis for *Kitligand* gene for all traits is found in appendix 4. The most significant value for *Kitligand* gene on face markings were found for HTG30 when white lower lip and blaze were compared to little white and no white with regards to color, p-value 0.0001 which is highly significant. The highest p-value for *Kitligand* gene on face markings was HTG30 when white lower lip, blaze, little white and no white were analyzed separately regardless of color, p-value 0.74 which is not significant.

The most significant value for *Kitligand* gene on leg markings were found for HTG30 when 0-1, 2-3 and four legs with white markings were analyzed with regards to color, p-value 0.0003 which is highly significant. The highest p-value for *Kitligand* gene on leg markings was TKY319 when 0-1, 2-3 and four legs with white markings regardless of color were analyzed, p-value 0.84 which is not significant. Most p-values for *Kitligand* gene markers on white leg markings did not show a significant association.

The lowest p-value for *Kitligand* gene on belly spot were found for HTG30, p-value <0.0001 which is highly significant. The highest p-value for *Kiligand* gene on belly spot was TKY319, p-value 0.88, which is not significant. One of the markers for *Kitligand* gene show significant association with the belly spot phenotype.

Pax3 gene

The full table with the results from the Contig analysis for *Pax3* gene for all traits is found in appendix 5. The most significant value for *Pax3* gene on face markings were found for NVHEQ82 when white lower lip and blaze were compared to little white and no white were with regards to color, p-value 0.007 which is significant. The highest p-value for *Pax3* gene on face markings was TKY1001 when white lower lip, blaze, little white and no white were analyzed separately with regards to color, p-value 0.55 which is not significant. Most p-values for *Pax3* gene markers are not significantly associated with white markings in the face (p>0.05)

The most significant value for *Pax3* gene on leg markings were found for COR10 when 0-1, 2-3 and four legs with white markings were analyzed with regards to color, p-value 0.046 which is just significant. No other p-values were significant for *Pax3* gene with white leg markings. The highest p-value for *Pax3* gene on leg markings was NVHEQ82 when 0, 1, 2, 3 and 4 legs with white markings were analyzed separately with regards to color, p-value 0.90 which is not significant.

The lowest p-value for *Pax3* gene on belly spot was found for TKY1001, p-value 0.47 which is not significant. The highest p-value for *Pax3* gene on belly spot was COR10, p-value 0.89 which is not significant either. None of the p-values for *Pax3* gene were significant for belly spot.

Association study in Icelandic horses

The Icelandic horses showed another distribution for HTG30 and the belly spot compared to the SWB, allele O seems to be associated with belly spot. LEX57 was also interesting in this trait, there allele R is the most common allele but shows an altered pattern with this trait.
In facial markings the Icelandic horses showed an altered distribution of alleles in TKY353 were it was more common with allele M than other alleles.

Linkage study

The linkage study did not provide any significant results at all between the markers and the traits. However it did show significant LOD-scores for some other markers. Table 8 shows the LOD-scores achieved when the markers are run against each other. All marker pairs in the table should show significant LOD-scores since they are located on the same chromosome. Significant LOD-scores are above 3.0.

Table 8. The LOD scores between the different markers.

Marker1	Marker2	LOD-score
LEX57	TKY353	0.00
LEX57	ASB23	1.51
TKY353	ASB23	1.20
COR10	NVHEQ82	5.43
COR10	TKY1001	2.15
NVHEQ82	TKY1001	3.64
HTG30	TKY319	1.17

Discussion

Since the phenotype of interest in this study is difficult to distinguish from similar but distinct phenotypes, both Icelandic horses and the SWB's were used for the study. Among the Icelandic horses, splashed white as well as other non-solid colors are accepted. Among the SWB non-solid colors are rare and it is therefore unlikely that another coat color would be misinterpreted as splashed white. Still there are a few horses that are registered as SWB that show rare patterns similar to the splashed white in Icelandic horses. One of the SWB offspring in this study looks like a minimally colored splashed white. This combined with the results from the association study has increased our belief that the splashed white coat color in Icelandic horses most likely has a counterpart among the SWB.

Inheritance study SWB

The inheritance study which included a total of 2265 horses provided important information regarding the genetic background for white markings in the SWB population. The dams of the foals were used as a control group to represent the average population and they showed a great variety of colors and markings. The group of offspring showed a greater frequency of white

markings on the face and on the legs. The frequency of belly spot did not differ so much from the control group.
71% of the offspring had a blaze whereas the same proportion for the dams was 44%; blaze is twice as common in the offspring group as within the group of dams. This implies that the stallions have increased the frequency of blaze in the offspring group compared to the population. Blaze is rather common in both groups. Some offspring do not have a blaze even though both parents have blaze. It is enough with one parent showing the trait. There are no cases of offspring having a blaze when neither of the parents has white markings on the face. The hypothesis after this study is that blaze has a multigenic background but is mainly controlled by one or few major genes. White lower lip was also twice as common among the offspring as in the control group. The main part of the offspring with a white lower lip has at least one parent with a white lower lip, but in some cases none of the parents have a white lower lip although the offspring does. The hypothesis from this study is that white lower lip most likely is a complex trait but regulated by few major genes.
Leg markings showed an altered distribution among the offspring compared to the mares. 32% of the offspring had four white legs but only 12% of the mares. 8% of the offspring had no white legs but 52% of the mare's had no white legs. This strongly implies that when at least one parent has more white legs there is a higher probability that the offspring will have more white on the legs. The hypothesis regarding the genetic background to leg markings is multigenic and that this phenotype is regulated by more genes than blaze and white lower lip.
The belly spot did not show any high difference between the two groups. This is probably caused by the fact that it is a very rare characteristic in both groups. There is another complication with this phenotype, i.e. it is not always reported. This is not a desired phenotype and during this study we have found examples that this is not always reported. This means that the frequency can be higher in both groups, just one group or maybe not higher at all. For the association study and the linkage analysis, most of the insecure phenotypes have been completed, but for the inheritance study where 2265 horses were included that was not an alternative.
The hypothesis regarding the genetic background to belly spots is that it shows a complex inheritance that involves several genes. There is most likely a multigenic background to the belly spot in SWB since the frequency is very low. The other alternative is that the allele is very rare.
Further studies are desired to determine the mode of inheritance for splashed white horses in Icelandic horses since this has not been looked into in this study.

Association study

The *Kit* gene markers show high association with both head and leg markings. TKY353 (also a *Kit* gene marker) shows the second highest association with belly spot. High significance in the association study indicates that the *Kit* gene is likely to be involved in the absence of pigmentation in these horses. Since Kit is important for migration and survival of the melanocytes this is not surprising. When the horses were grouped according to the theories regarding mode of inheritance the significance increased. This could indicate that the main mode of inheritance is understood although it is highly likely that there are more genes involved in the extension of the white patterns. The high significances found in this study and the fact that the significance increased in some cases when the number of alternative phenotypes was reduced shows that there is an association between the *Kit* gene and white markings in horses. Since the *Kit* gene is one of the important genes during melanocyte development and migration during embryo development this is not unexpected.

HTG30 is the only marker with significant results in association with the belly spot. In there is primarily allele R which is responsible for the high chi-square values. After investigating the individuals with allele R that have not been noted as having a belly spot they appear to have extensive white markings on other places of the body and it is therefore highly possible that these individuals actually do have a belly spot that was not noted during the identification of the horse. Some of these horses have had completed phenotypic records, whereas some of them appear not to have a belly spot. It is important to keep in mind that this marker is just a marker for the *Kitligand* gene and it does not necessarily represent a mutation in the *Kitligand* gene. More interesting facts are that this particular allele just exists in some of the families, and in the other families allele O seems to have a similar function. It is possible that there are different alleles representing the same mutation that give rise to this phenotype since the microsatellites are just markers for a gene and recombination between markers and a causative mutation could have occurred. A further study of the area between HTG30 and *Kitligand* gene, using microsatellites or SNP's, to reveal a possible higher association is desirable, for more information see further studies.

In the Icelandic horse half sib families that have been studied the allele O in HTG30 seem to be associated to the splashed white phenotype, the individuals believed to be heterozygous for splashed white are heterozygous and one of the individuals that should be homozygous is in fact homozygous. The limited number of Icelandic horses that are included in the study made it difficult to get results from the association test. One of the splashed white Icelandic horses has the same genotype in the markers as one of the SWB stallions with belly spot. They had the same genotype in four markers and shared one allele in three of the other markers. In some of the markers the alleles that they had in common were rare alleles in the SWB. It is not possible to say if the alleles were rare in the Icelandic horse as well due to the limited material.

When the Icelandic horses and the SWB were compared, there seemed to be similarities between the splashed white and the belly spot. One of the SWB stallions had the same phenotype as one of the Icelandic horses with splashed white. It is also interesting that there seem to be a haplotype involved in the splashed white and belly spot phenotype. When several markers are studied at the same time a pattern is possible. Unfortunately there was no time for a haplotype analysis. The haplotype analysis is an important part of a further study in this project.

Linkage analysis

The linkage analysis did not provide any significant results; this does not mean that there is no linkage between the phenotypic characters and the markers. In the linkage analysis software which was used (Crimap) it is necessary to determine whether the individuals are heterozygous or homozygous. Also Crimap only works when the phenotype show simple Mendelian inheritance which these phenotypes do not appear to have.

The most likely cause to the lack of results from the linkage analysis is that we are not dealing with a trait that shows simple Mendelian inheritance and that we have not been able to find the heterozygous and homozygous individuals yet. There are other programs that work with traits that do not show the simple Mendelian inheritance but the lack of time in this project did not allow for further analysis with other programs. The difference in results between the association study and the linkage analysis study probably reflect the fact that the mode of inheritance is not relevant for the association analysis but highly relevant for the linkage analysis.

During the association study as well as the primary linkage analysis, the Icelandic horses and the SWB were separated since it is not known if there is a difference between the two breeds, regarding color patterns.

Conclusion

The phenotypic study showed an interesting inheritance of markings from the stallions to their offspring. Some of the stallions had a larger proportion offspring with extensive markings than the others. This implies that there is a genetic background to this phenotype. In some of the half-sib families there are strong differences between the proportions of offspring with different white markings. The facial marking is one example, where the stallions with more extensive white patterns produce offspring that have more white facial markings.

These characteristics are similar to the ones for splashed white, and this is why it is important to determine the differences. In another ongoing study the same horses that have been used for this study have been characterized for the sabino patterning and genotyped. Two of the stallions included in the material are slightly roaned.

The first hypothesis in this study was that there are several genes affecting the belly spot and splashed white coat color, also that it would be the same genes in both SWB and Icelandic horses. From this study we draw the conclusion that several genes most likely affect the coat colors of interest. The same genes seem to be involved. We cannot dismiss the first hypothesis. From the frequencies of the phenotypes it is likely that the alleles are recessive or rare or a combination. The *Kitligand* gene is likely to be one of the major genes controlling these phenotypes.

The second hypothesis was that horses with belly spot (SWB) or horses that show splashed white (Icelandic horses) are homozygous while the heterozygous individuals show extensive white markings. From this study it is not possible to say that the individuals with belly spot or splashed white coat color are homozygous but the frequencies of horses with those traits and the most likely inheritance pattern indicate that horses with belly spot are homozygous.

The third hypothesis was that chestnut colored horses would show more extensive white markings. White markings are more extensive among chestnuts than bay or black horses. This was found both among the offspring and among the control group.

White markings in the face seem to have one major gene controlling, the *Kit* gene, where white lower lip is most likely recessive and blaze is dominant. There are probably several genes controlling the extension of white markings in the face. Leg markings are probably also the result of several genes interacting; the *Kit* gene is likely to be involved. The *Pax3* gene is unlikely to be involved in the phenotypes studied in this study.

Further studies

Further studies in this area are needed to sort out the genetics behind these traits. It would give more information to perform an inheritance study that would provide the true inheritance patterns for the traits. For this further information regarding relatives is needed.

One study that would probably give important information is a pure case-control study where more microsatellites and/or SNP's would be studied. SNP's would be preferred to create a better map of the area surrounding the *Kitligand* gene. To make a detailed map the SNP's are better since they are more frequent in the genome, there are much less microsatellites in the genome. In order to come closer to the gene the SNP's would be better. If SNP's are used to cover the area surrounding the most interesting genes *Kit* and *Kitligand* a haplotyope analysis of the results would probably provide much information.

Another study which would also provide more information is to test the included individuals for chestnut. It seems that there is a connection between color and white markings. Chestnut horses seem to have more white markings and the association analysis gave more significant p-values when the colors were separated.

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Appendix

1. PCR-protocol

All volumes are given in µl. The (p) or (m), after the name of the fluorescent dye which has been used, indicates if the forward primer (p) or the m-13 linker (m) has been labeled.

Marker	**LEX57**	**HTG30**	**TKY353**	**COR10**	**NVHEQ82**	**ASB23**	**TKY1001**	**TKY319**
Labeled with	NED (m)	FAM (m)	VIC (m)	VIC (m)	VIC (p)	VIC (p)	NED (p)	NED (p)
10X Buffer	1,0	1,0	1,0	1,0	1,0	1,0	1,5	1,5
dNTP (20µM)	0,1	0,1	0,1	0,1	0,1	0,1	0,1	0,1
AT Gold (5 U/µl)	0,1	0,1	0,1	0,1	0,1	0,1	0,1	0,1
Primer F (10 µM)	0,1	0,1	0,1	0,1	0,1	0,5	1,0	0,5
Primer R (10 µM)	0,5	0,5	0,5	0,5	0,1	0,5	1,0	0,5
M13 linker (10 µM)	0,5	0,5	0,5	0,5	0,0	0,0	0,0	0,0
ddH_2O	6,7	6,7	6,7	6,7	7,6	6,8	10,3	11,3
DNA (various concentrations, see Materials and Methods.	1,0	1,0	1,0	1,0	1,0	1,0	1,0	1,0
Total	10,0	10,0	10,0	10,0	10,0	10,0	15,0	15,0

2. PCR-program

1. Program for LEX57, HTG30 and TKY353

95 °C	10 min	
95 °C	30 s	
65 (-1) °C	30 s	x14 cycles
72 °C	30 s	
95 °C	30 s	
52 °C	30 s	x30 cycles
72 °C	30 s	
72 °C	7 min	

2. Program for COR10.

96 °C	10 min	
95 °C	30 s	
60 (-0,5)°C	1 min	x20 cycles
72 °C	1 min	
95 °C	30 s	
57 °C	30 s	x20 cycles
72 °C	1 min	
72 °C	10 min	

3. Program for NVHEQ82

96 °C	10 min	
95 °C	30 s	
65 (-1) °C	30 s	x14 cycles
72 °C	30 s	
95 °C	30 s	
60 °C	30 s	x30 cycles
72 °C	30 s	
72 °C	10 min	

4. Program for ASB23.

95 °C	10 min	
95 °C	30 s	
58 °C	1 min 30 s	x34 cycles
72 °C	1 min	
72 °C	60 min	

5. Program for TKY1001.

90 °C	5 min	
94 °C	1 min	
55 (-1) °C	30 s	x6 cycles
72 °C	30 s	
94 °C	45 s	
50 °C	30 s	x27 cycles
72 °C	30 s	
72 °C	30 min	

6. Program for TKY319 and TKY18.

94 °C	5 min	
94 °C	45 s	
65 (-1) °C	45 s	x14 cycles
72 °C	45 s	
94 °C	45 s	
52 °C	45 s	x31 cycles
72 °C	45 s	
72 °C	10 min	

3. Results from Conting analysis on *Kit* gene markers.

Face markings

	A			B			C			D		
	Chi-square	df	p-value	Chi-square	df	p-value	Chi-square	df	p-value	Chi-square	df	p-value
TKY353	45,73	20	8,78E-04	27,83	8	5,08E-04	39,02	12	1,05E-04	22,82	4	1,38E-04
LEX57	94,72	30	1,24E-08	11,21	2	5,11E-01	89,36	18	0,00	5,88	6	4,36E-01
ASB23	53,68	30	4,99E-03	33,12	2	9,27E-04	43,08	18	7,79E-04	25,31	6	2,99E-04

White leg markings

	A			B			C			D			E			F		
	Chi-square	df	p-value	Chi-square	df	p-value	Chi-square	df	p-value	Chi-square	df	p-value	Chi-square	df	p-value	Chi-square	df	p-value
TKY353	59,70	36	7,79E-03	28,06	6	3,11E-02	43,63	20	1,69E-03	20,33	8	9,17E-03	38,65	20	7,38E-03	16,58	8	3,48E-02
LEX57	112,46	54	5,39E-06	20,04	4	6,95E-01	105,99	30	0	14,31	2	2,82E-01	99,86	30	0,00E+0 0	8,90	2	7,12E-01
ASB23	69,26	54	7,89E-02	37,20	4	4,18E-02	42,94	25	1,42E-02	19,27	2	8,21E-01	48,87	25	2,93E-03	21,12	2	4,86E-02

Belly spot

	Chi-square	df	p-value
TKY353	7,14	4	1,29E-01
LEX57	5,59	6	4,70E-01
ASB23	5,18	6	5,21E-01

4. Results from Conting analysis on *Kitligand* gene markers.

Face markings

	A			B			C			D		
	Chi-square	**df**	**p-value**	**Chi-square**	**df**	**p-value**	**Chi-square**	**df**	**p-value**	**Chi-square**	**df**	**p-value**
HTG30	56,28	30	2,54E-03	8,61	12	7,36E-01	48,75	18	1,16E-04	5,52	6	4,78E-01
TKY319	27,60	30	5,91E-01	8,42	12	7,51E-01	16,36	18	5,67E-01	5,65	6	4,63E-01

White leg markings

	A			B			C			D			E			F		
	Chi-square	**df**	**p-value**	**Chi-square**	**df**	**p-value**	**Chi-square**	**df**	**p-value**	**Chi-square**	**df**	**p-value**	**Chi-square**	**df**	**p-value**	**Chi-square**	**df**	**p-value**
HTG30	84,15	54	5,38E-01	36,99	24	4,39E-02	23, 00	12	2,77E-02	15,01	12	2,41E-01	64,39	30	2,62E-04	54,73	30	3,81E-03
TKY319	50,68	54	6,03E-01	26,82	24	3,13E-01	11,43	12	4,92E-01	10,99	12	5,30E-01	23,11	30	8,11E-01	22,47	30	8,36E-01

Belly spot

	Chi-square	**df**	**p-value**
HTG30	31,11	6	2,40E-05
TKY319	2,36	6	8,84E-01

5. Results from Conting analysis on *Pax3* gene markers.

Face markings

	A			B			C			D		
	Chi-square	df	p-value	Chi-square	df	p-value	Chi-square	df	p-value	Chi-square	df	p-value
COR10	28,92	30	5,22E-01	11,89	12	4,54E-01	18,16	18	4,45E-01	5,17	6	5,22E-01
NVHEQTKY1001	38,93	25	3,74E-02	9,55	10	4,81E-01	31,68	15	7,12E-03	7,66	5	1,76E-01
	28,44	30	5,47E-01		12			18			6	

White leg markings

	A			B			C			D			E			F		
	Chi-square	df	p-value	Chi-square	df	p-value	Chi-square	df	p-value	Chi-square	df	p-value	Chi-square	df	p-value	Chi-square	df	p-value
COR10	68,19	54	9,27E-02	33,44	24	9,52E-02	16,18	12	1,83E-01	12,16	12	4,33E-01	44,14	30	4,63E-02	34,19	30	2,73E-01
NVHEQTKY1001	33,43	45	8,98E-01	18,97	20	5,24E-01	10,84	10	3,70E-01	12,81	10	2,35E-01	19,25	25	7,85E-01	21,13	25	6,85E-01
	59,18	54	2,92E-01	26,98	24	3,06E-01	13,79	12	3,14E-01	8,67	12	7,31E-01	36,70	30	1,86E-01	27,97	30	5,72E-01

Belly spot

	Chi-square	df	p-value
COR10	2,35	6	8,85E-01
NVHEQTKY1001	2,71	5	7,45E-01
	5,55	6	4,76E-01