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Genetics of gaits in Icelandic horses

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Contents

1. Abstract

A genome-wide association analysis of Icelandic horses has shown that a mutation in *DMRT3*, doublesex and mab-3 related transcription factor 3 (‘gait keeper’ mutation), has a strong impact on gait pattern. The *DMRT3* mutant allele (A) has been found in high frequency in gaited breeds, and breeds used for harness racing, while non-gaited breeds were homozygous for the wild-type allele (C). Icelandic horses have the ability to perform alternate gaits besides the three basic gaits (walk, trot, canter/gallop) such as flying pace and tölt. Homozygosity for the *DMRT3* nonsense mutation enables pace and has a positive effect on the quality of tölt (beat quality, speed capacity and suppleness). CA Icelandic horses on the other hand have better scores for walk, trot, canter and gallop. Previously, two border-line significant single nucleotide polymorphisms (SNPs) have been associated with tölt in another genome-wide association study (using breeding values as the phenotype). The aim is to validate the two tentative associations in additional Icelandic horses. In this study we want to confirm the association of two SNP markers, one on Chromosome 13 and one on Chromosome 28 with breeding values (EBVs) for tölt (tölt, slow tölt and direct tölt). We also want to investigate the genotype frequencies in; American Curly, American miniature horse, American Saddlebred, Friesian, Hackney, Morgan, Peruvian Paso, Rocky Mountain and Swedish Warmblood. TaqMan SNP Genotyping Assays was used to genotype the individuals for the SNP markers of interest. The association was confirmed for EBVs on Chromosome 28 (tölt P=0, 03*, P=0, 03*) and also Chromosome 13 (tölt P=0.009**). Next step is to fine map the confirmed genomic regions.

Keywords: Gaits, SNP, EBV, Tölt,

2. Introduction

The Horse (*Equus caballus*) and has played a vital role for human civilization in regards to transportation, exploration, warfare and racing. The domestic horses spread soon after the initial domestication (5500 years ago) worldwide during the Bronze Age, most likely through horseback riding and migration by humans. After that, the process of gene flow/exchange between wild and domesticated individuals (Cieslak et al., 2010) has continued. Modern maternal lineages contain a significant proportion of diversity that was present during the time of domestication (Petersen et al., 2013). A study conducted by Lindgren et al., (2004) indicates that a limited number of stallions might be a traditional breeding practice, maybe even when the wild horses were roaming free, and that could explain the low diversity. The current number of horse breeds today is around 500 (Petersen et al. 2013a). Most breeds are determined by a set of standardised regulations determined by the breeds association but not all breeds are closed populations. Earlier studies indicated that coat color and coloration pattern was an early-targeted phenotype and it is still very popular (Ludwig et al., 2009). Petersen et al., (2013a), performed the first large scale study in horses on how selective pressure on characteristics such as coat color, muscle mass and performance gait (pattern of locomotion) has effected different breeds. Traits commonly selected upon are; size (American Miniature Horse or large horses) muscle capacity (draft horses), gaits and populations that are unmanaged (landrace populations). Breeding in the past has focused on preserving and improving traits connected to aesthetics and performance. However the range between different breeds is quite apparent when you compare Miniature horse (0.74m and 100 kg) and draft horses (1.8m and 900 kg) plus that there is a great variation within the breeds (Petersen et al., 2013a).

Locomotion of the horse is something that has been in the interest of humans since the time of Aristotle (384-322 BC) (Smith & Ross 1910). Attempts to analyse and define different gaits of horses were made in 1779 by Goiffon and Vincent, who attached bells with different tones to the hooves (Leach & Dagg., 1983). Muybride (1887) was another scientist who used series of cameras in order to capture the movement of the horse (Leach & Dagg., 1983). Economic factors are a major motivator in regards to encouraging development of early performance evaluation, in order to improve the training and selection of young horses (Barrey, 1999). Performance abilities such as racing performances are other phenotypes that have been quite lucrative for human interest. Certain breeds have proven suitable for different distances; short (American Quarter Horse), intermediate (Thoroughbred horses) and long distances (Arabian and Akhal Teke). Myostatin gene (*MSTN*) which is a negative regulator of muscle development has become associated with racing suitability (Tozaki et al 2011, Hill et al., 2010). An intronic variant in *MSTN* is predictive of the best race

distance for the Thoroughbred, Hills et al.,(2010) and suggests that horses homozygous for the “C” allele are better suited for short distance racing, heterozygotes are more capable middle-distance racers and homozygotes for the “T” allele have a greater stamina for long-distance race. In addition to predicting optimal racing distance, *MSTN* has been considered as an important gene to racing success and also a role in body composition (Tozaki et al 2011).

The definition of a gaited horse is a horse that can perform a gait with a different footfall pattern than showed in walk, trot and canter/gallop. The identification by Petersen et al (2013a) of a locus under strong signal of selection on ECA23 was apparent for all “gaited breeds”. This was considered to be a significant cross-breed signal of selection and conserved haplotype on ECA23 common among gaited breeds.

2.1 Gaits in horses

A study published in 2012 by Anderson et al., discovered that a nonsense mutation in *DMRT3* was present at a high frequency in gaited breeds and; horses bred for harness racing. It has a strong impact on gaitedness in horses. The ability to perform alternate gaits is under heavy selection and is often a breed-defining characteristic. The common gaits for the majority of domestic horses and other wild equids are walk, trot, canter and gallop. Some horses have the ability to also perform additional gaits, such as pace. Pace is a gait where the horse move in a two-beat ipsilateral gait, compared to trot where the horse move in a two beat contralateral gait (Petersen et al., 2013). The gaits can be determined by the symmetry and dissymmetry of the limb movement (Barrey, 1999). Symmetric gaits would be; walk, tölt, pace and trot, Asymmetric gaits would be; canter/ gallop. Within each gait there are more variations due to speed and pattern. Dressage competitions are largely made up on performing different gaits with different speed and with collected versus extended leg strides.

Promerova et al., (2014) looked into the worldwide distribution of the *DMRT3* nonsense mutation and constituted that the mutation is not restricted to a certain geographical area. Breeds with a higher frequency of the stop mutation are either considered to be gaited, or bred for harness racing. Icelandic horses can perform the gait tölt besides the three common gaits; walk, trot and canter/gallop, and many horses also have the ability to perform a fifth gait called pace. The individuals that can perform pace are classified as five gaited and the ones that perform tölt are called four gaited. Tölt has similar foot pattern as walk, where one or two hoofs are simultaneously in contact with the ground and is therefore without a suspension phase. Tölt can be ridden in a slow pace as walk or in a fast tempo.

2.2 Estimated breeding value (EBV)

The global database for Icelandic horses, Worldfengur, contains over 410 000 individuals where pedigree, offspring, estimated breeding values (EBVs) and competition results are available (worldfengur.com). Valued breeding horses are those that have a high estimated breeding value and those who perform well at breeding field tests. Breeding values are estimated from breeding field test scores and close relatives performance. Breeding is generally based on preferable breeding values. Preselection of individuals that partakes in the breeding evaluation has led to increasingly higher average total scores over time, a result which can be partly explained by selective breeding (Albertsdóttir et al., 2011), where the offspring perform better than their sires. However it is a strong indication that horses are chosen to attend breeding field tests based on their assumed potential to achieve high scores. Only a small part of registered horses attend these field tests, so it can be assumed that these are not a random sample of the population. The phenotypes assess in this project are; EBV Tölt, EBV Slow and Direct tölt. Both EBV values are a combination of individual competition scores and scores derived from heritable performance and direct tölt are values only derived from individual competition scores.

3. Aim

The aims of this project are:

- Confirm or reject the association of two SNP markers on Chromosome 13 and Chromosome 28 with EBVs for tölt in Icelandic horses.
- Analyse EBV tölt, EBV slow tölt and direct tölt in the different genotypes for the two genetic markers identified in Icelandic horses.
- Investigate the genotype frequencies of the two SNPs in American Curly, American miniature horse, American Saddlebred, Friesian, Hackney, Morgan, Peruvian Paso, Rocky Mountain and Swedish Warmblood.

4. Material & Methods

4.1 Prior experiments

A genome wide association analysis study (GWAS) was conducted prior to this study (Shresta et al unpublished) with 119 horses that were also used in this study. 50 horses of these 119 were re-genotyped for this thesis in order to confirm the GWAS results.

4.2 Horse material

DNA samples from Icelandic horses were genotyped for the two SNP's Chromosome 13:22296211 bp (345 horses) and Chromosome 28:35485344 bp (348 horses) of interests. 119 of these horses were included in the initial GWAS. 368 horses of different breeds; American Curly (n=85), American miniature horse (n=35), American Saddlebred (n=32), Friesian (n=8), Hackney (n=32), Morgan (n=58), Peruvian Paso (n=7), Rocky Mountain (n=28) and Swedish Warmblood (n=83) were also genotyped in order to analyse the potential association between typical gait and leg action in the different breeds and allele frequencies. The DNA samples (extracted prior from blood and hair) were collected from the biobank at the Animal Genetics Laboratory, Swedish University of Agricultural Sciences, Uppsala, Sweden.

4.3 Different traits analysed

The phenotypic traits of the Icelandic horses were collected from Worldfengur database. A range of different EBV traits (body composition and ridden abilities/gaits) gathered from FIZO (breeding rules for Icelandic horses) were analyzed, plus an average from tölt competition scores here named Direct tölt. The phenotypes (EBV tölt, EBV slow tölt and direct tölt) were compared with the different genotypes for both SNP's.

The phenotypes assessed in this project are:

EBV Tölt – Even 4-beat rhythm with long strides in front and behind, elegant lift and action of the front legs, movements extremely flexible and supple. (FIZO)

EBV Slow Tölt – Even 4-beat tölt with long strides in front and behind, lots of lift and action of the front legs, movements extremely flexible and supple. (FIZO)

Direct tölt – an average of scores from tölt competitions.

4.4 Isolation of DNA and SNP genotyping

Deoxyribonucleic acid had been prepared prior from the hair root using a standard hair-preparation procedure or blood using a standard blood-preparation procedure. One hundred microliters Chelex 100 Resin (Bio-Rad Laboratories, Hercules, CA) and 7 µl of proteinase K (20 mg/mL; Merc KgaA, Darmstadt, Germany) were added to the sample. The mix was incubated at 56℃ for 1 h and the proteinase K was inactivated for 10 min at 95 ℃. For DNA preparation from blood samples (also done prior to this study), 350 µl of blood was used and isolated by the Qiasymphony instrument (Qiagen, Hilden, Germany). The samples were genotyped for two SNP markers Chromosome13:22296211 bp and Chromosome28:35485344). SNP

genotyping was performed with the StepOnePlus Real-Time PCR System (Life Technologies [Thermo Fisher Scientific], Waltham, MA) using custom designed TaqMan SNP Genotyping Assays (Applied Biosystems by Life Technologies [Thermo Fisher Scientific]) as previously described (Jäderkvist et al., 2014; Andersson *et al*, 2012; Promerová et al., 2014).

4.5 Statistical Analysis

The statistical analyses were performed in the software: R (R Development Core Team, 2005), and Simple Interactive Statistical Analysis (SISA; Quantitative Skills, 2013). For comparing phenotypic trait means with genotype Student's t-test was used.

5. Results

5.1 Allele frequency

Table 1. Allele frequency distribution for SNPs on chromosome 13 and chromosome 28. Allele A is the major allele with 80 % for the SNP on chromosome 28. The dominating allele for chromosome 13 was allele A with 60 %.

	Allele Frequency	
Genotypes Chr 28	Allele A	Allele G
	0,8	**0,2**
Genotypes Chr 13	Allele A	Allele C
	0,6	**0,4**

Table 2. P-values derived from Students T-test presenting prior results combined with new from this thesis. Shresta et al were genotyped through GWAS prior to this study. Axling et al were genotyped for this study. Combined results represent results from prior study as well as newly genotyped individuals. ** = P<0.001, P<0.05

Tölt	Chr 13			N	Chr 28			N
	AA/AC	AC/CC	AA/CC		AA/AG	AG/GG	AA/GG	
Shresta et al	0.001**	0.184	0.005*	119	0.202	0.165	0.05*	119
Axling et al	0.0094**	0.073	0.827	226	0.032*	0.201	0.034*	229
Combined	0.898	0.541	0.592	345	0.274	0.397	0.173	348
Slow Tölt								
	AA/AC	AC/CC	AA/CC		AA/AG	AG/GG	AA/GG	
Shresta et al	0.3944	0.209	0.115	119	0.303	0.138	0.863	119
Axling et al	0.0816	0.024*	0.279	226	0.208	0.299	0.075	229
Combined	0.4529	0.093	0.182	345	0.146	0.334	0.087	348

For this thesis 229 Icelandic horses were genotyped for chromosome 28 and 226 Icelandic horses were genotyped for chromosome 13 and matched with relevant phenotype. The findings from Shresta et al (Table 2) where genotyped prior in connection to the GWAS study mentioned above. When results from both Shresta et al., and Axling et al., was combined the significant results were not confirmed (Table 2). In order to confirm prior GWAS results 50 horses were re-genotyped of the 119 used. Different genotype combination was significant for the different population samples. Shresta et al had a significant result for AA/CC on chr 13 for tölt. Axling et al had significant result for AA/AG on chr 28 for tölt, and AC/CC on chr 13 for slow tölt.

5.2 SNP on Chromosome 28

For the SNP on Chromosome 28, 229 new horses were used in the analysis; 140 homozygous AA horses, 78 heterozygous AG horses and 11 homozygous GG horses. The result show that there was a significant difference in EBVs for tölt (AA vs AG P=0,032**, AA vs GG P=0, 05* (Table 2 & Figure 1)) and non- significant for slow tölt between the different genotypes (Table 2 & Figure 2).). See figure 1 and 2.

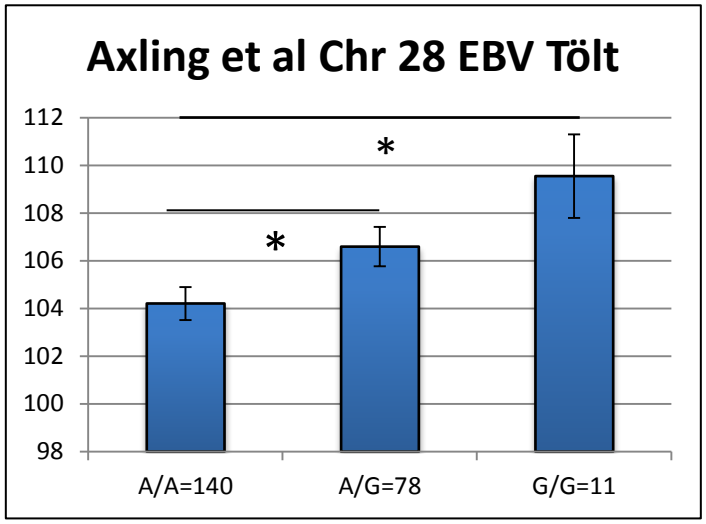

Figure 1. EBVs for tölt for the different genotypes. The error bars is represented by SE. *= P<0,05

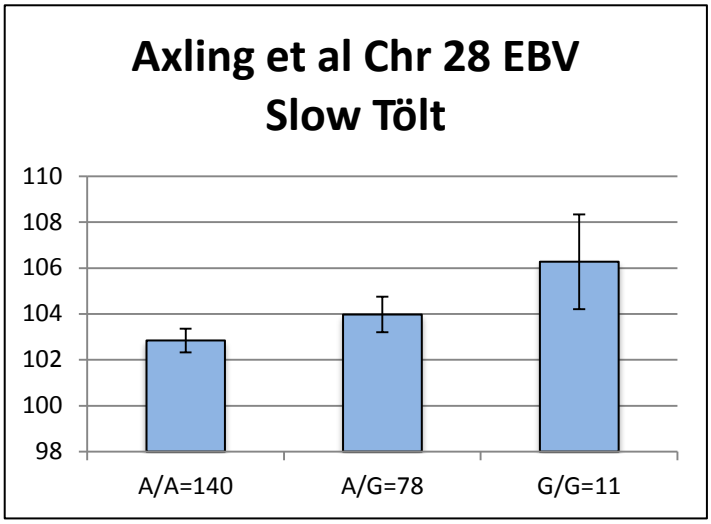

Figure 2. EBVs for slow tölt for different genotypes. The error bars is represented by SE. *=P<0,05

5.3 SNP on Chromosome 13

For the SNP on Chromosome 13, 226 horses were used in the analysis with; 92 homozygous AA horses, 99 heterozygous AC horses and 35 homozygous CC horses. EBV's for tölt between the genotypes were significant for Chromosome 13 (AA vs AC P=0.009** Table 1 & Figure 3) when only the new horses (Axling et al) were genotyped however the significance was not confirmed with all 345 horses.

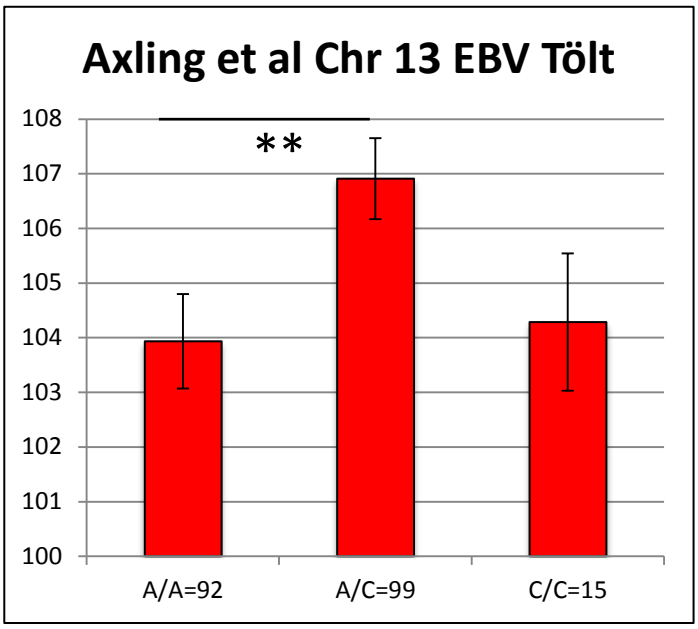

Figure 3. EBVs for tölt for the different genotypes. The error bars is represented by SE. **= P<0,001

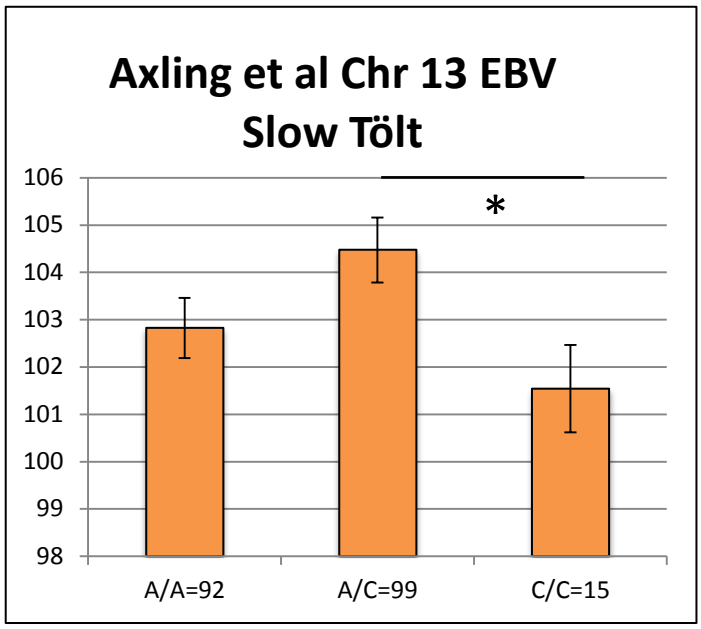

Figure 4. EBVs for slow tölt for the different genotypes. The error bars is represented by SE. *= P<0,05

5.4 Direct tölt

This phenotype showed no significant results between the individual horses tested. The analysis was only conducted on the SNP for chromosome 13 and could not be compared with results from the prior GWAS study due to poor competition participation of the horses in the sample.

5.5 Allele frequency distribution in different breeds

Table 3 shows other breeds tested for the SNP marker on Chromosome 13, with percentage in regards to the genotypes. Information about gaitedness comes from Promerova et al., (2014). Gaited= the breed is considered gaited; some = a fraction of the horses of this breed are gaited; not gaited, gaited horses are not observed for this breed; harness, breed used for harness racing.

Table 3. Genotypes for the breeds tested, the gaited status and allele frequency (%).

Breed	AA %	AC%	CC%	Total n	Gaited	Allele frequency A
American Curly	25	44	32	85	Some - foxtrot	0.46
Morgan	17	47	36	58	Some – singlefoot	0.41
Rocky Mountain	7	25	68	28	Gaited – singlefoot, rack	0.19
American Miniature horse	57	31	11	35	Some – pace	0.72
Peruvian Paso	29	57	14	7	Gaited – paso	0.57
American Saddlebred	16	44	41	32	Some- rack	0.37
Friesian	0	63	38	8	Not gaited	0.31
Hackney	9	44	47	32	Harness	0.31
Icelandic horses	92	99	15	345	Gaited- tölt,pace	0.6
Swedish Warmblood	55	41	4	83	Not gaited	0.75

Rocky Mountain showed a preference for the C allele with only 20 % having the A allele (Table 3). Friesian had no "AA" horses and 68% being AC heterozygous, however results from only 8 individuals (Table 3). Swedish Warmblood had a high A allele frequency with 75% (Table 3).

6. Discussion

The association previously detected in the GWAS study (Shresta et al., unpublished) was confirmed for the SNP marker on Chromosome 28 of one phenotypes (EBV tölt, Figure 1) and on chromosome 13 for one phenotype (EBV slow tölt, Figure 4). The implications of this could be that the areas involved are affecting the quality of tölt. The parameters involved when determining tölt is; good speed, clear beat, high action, long strides, big movements and suppleness (FIZO). One hypothesis was that one of the genotype would prove to be more beneficial in order to achieve better quality of tölt, and this was seen for Chromosome 28 with homozygous GG horses performing better and in chromosome 13 where AC horses performed better. However further individuals would be preferred in order to see if the significant pattern remains for chromosome 28.

The phenotype for the different genotypes when looking at *DMRT3* in Icelandic horses is associated with gaitedness and performance, AA enables the ability to pace and also to achieve high scores for tölt (Kristjansson et al., 2014). This appears to be due to superior speed capacity and suppleness of the AA horses compared with the CA horses. Both speed and suppleness has a great impact of the overall score for tölt (FIZO 2014). Anderson et al., (2012) showed that Icelandic horses with the genotype CA performed significantly higher for trot compared to homozygous mutant horses. CA horses performing significantly higher were also confirmed in a study of Kristjansson et al (2012), where it was also established that CA horses had a significantly higher scores for canter/gallop. These findings follows the pattern observed for the SNP on chromosome 13 for both Shresta et al and Axling et al, with AC horses performing significantly (Table 2) better. However when the two different population samples were combined the significance was lost, a deeper demographic analysis of the population is needed in order to determine why. In the previous genome wide association study conducted by Shresta (unpublished data) one Swedish population was compared with a Dutch population, there was a strong association on chromosome 28 for the Dutch population however not for the Swedish population. This might be due to different breeding focuses where different traits are emphasised and valued. Even though certain traits are standardized according to FIZO (2014), the evaluations for certain breeding field tests might be slightly different which might lead to different phenotypes to be more desired. This was not evaluated

in this thesis however it is of importance to take into account when comparing different populations that are generally considered to be uniform and standardized.

Other horse breeds were added in order to evaluate other phenotypes for the SNP markers. The different gaits between the breeds used in this study are quite different stride wise. Peruvian Paso performs a gait called paso llano, which is a 4 beat lateral gait speed wise between walk and canter. Paso llano compared to racking, which is most commonly associated with American Saddlebred, is also a 4 beat lateral gait however with longer strides and greater speed usually. Comparing different phenotypes of breeds like this is important in order to identify regions of the genome that are significantly different, and maybe genes with variations targeted by selective breeding. One hypothesis was that these SNP markers might reveal a pattern with different genotypes between the breeds or similar genotype for similar gaits. Further analysis comparing the different gaits is required to draw any conclusion from the genotypes. Unfortunately the gaits of the other breeds are not assessed in a similar way as tölt so there is no data to perform similar statistical analysis on, at this time. Soft gaited horses were probably more desired before carts and carriers were introduced. Then focus shifted to larger size in order to draft and trotting abilities (better suited gait for carriers or warfare). So there has most likely been an active selection for and against different gaits depending on the current preference. Considering the different morphology and traits desired in different horse breeds is interesting and important to investigate the different genetic pathways that make up the genome of the breeds.

6.1 Further research

To add further homozygous GG horses for the SNP chromosome 28. Further investigate the different populations and look into origin and sex. Also more analysis of neighbouring genes close to the SNPs. Investigate which alleles that drives the significance for the Icelandic horse but also for the other breeds. Analyse more SNPs that were significant from the prior GWAS study.

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