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THE ASSOCIATION OF TWO GENETIC MARKERS AND EVALUATION SCORES FOR TÖLT IN ICELANDIC HORSES

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The association of two genetic markers and evaluation scores for tölt in Icelandic horses

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CONTENTS

ABSTRACT

Previous studies have shown that some horse breeds carries a mutated variant of the gene *DMRT3,* which is crucial for horses to perform alternative gaits like pace and tölt. The mutation is also important for harnessing racing performance. A previous unpublished genome wide association study found that two SNP (single nucleotide polymorphism) markers on chromosome 13 (22,296,211 bp) and 28 (35,485,344 bp) were significantly associated with the estimated breeding values (EBV) for tölt. They could possibly have an effect on the quality of slow tölt and tölt. The aim of this current study was to follow up the previous findings in additional Icelandic horses. We used 95 five-gaited Icelandic horse mares that were all shown at a breeding field test between the ages of 5 and 6. Hair samples were selected from the Animal Genetics Laboratory, Swedish University of Agricultural Sciences, Uppsala, Sweden. The DNA was prepared with the Chelex procedure, and the horses were genotyped for two SNPs on chromosome 13 (22,296,211 bp) and chromosome 28 (35,485,344 bp). The SNP genotyping was performed with the StepOnePlus Real-Time PCR System using custom designed TaqMan SNP Genotyping Assays. When it came to genotype frequencies for the SNP marker on chromosome 13, the dominating allele was T with 64 % frequency. For the SNP marker on chromosome 28, the dominating allele was A with 83 % frequency. The study showed no significant associations between the SNP markers on chromosome 13 and chromosome 28 and the evaluation score of tölt and slow tölt.

INTRODUCTION

Gaited Breeds

Anderson et al. (2012) discovered that gaited horses carry a mutation in one of the genes expressed in the neurons in the spinal cord. The gene is called *DMRT3* and the mutation is crucial for the horses to be able to perform alternative gaits, such as tölt and pace. The breeds that can perform these gaits are called gaited breeds. In Anderson et al. (2012) study, it was found that three-gaited breeds, such as Swedish Warmbloods and Thoroughbreds, were homozygous wild-type (CC), while most gaited breeds, like the Icelandic horse, were homozygous AA or heterozygous CA. Andersson et al. (2012) genotyped 352 Icelandic horses and found that most of the horses evaluated as four-gaited (that could perform walk, trot, gallop and tölt) were CA, but 31 % of the horses had the AA genotype. All five-gaiters (that could perform walk, trot, gallop, tölt and pace) except one were homozygous AA. Thus for the ability to perform pace, the homozygosity for the *DMRT3* mutation is required (Anderson et al. 2012). However, even though the homozygous AA genotype is required for pace, it is not sufficient. Other factors, such as other genes, the maturity of the horse, and environmental effects, such as the type and quality of the training, may be more probable causes leading to an incomplete penetrance of the alternative gaits tölt and pace. Thus, the *DMRT3* mutation seems to be the one factor that makes it possible for horses to perform alternative gaits. This mutation is advantageous for the alternative gaits, but it may work against the normal gaits, walk, trot and canter/gallop. In the study by Andersson et al. (2012) it was found that Icelandic horses with homozygous mutants for AA had lower scores for gallop

and trot compared to horses without this mutant allele. Another study by Kristjánsson et al. (2014) confirmed the results from Anderson et al. (2012), it showed that the horses homozygous for AA had significant higher scores for tölt compared to the CA genotype, whiles the Icelandic horses with the CA genotype had significant higher scores for walk, trot and canter/gallop. Consequently, there are reasons for not selecting the mutation in non-gaited horses where the usual gaits are more important than the alternative gaits, such as for racing horses, dressage or show jumping horses (Andersson et al. 2012).

The Icelandic Horse

Petersen et al. (2013) defined a breed performing alternative gaits by characterizing the variations of the footfall timing and pattern, other than those shown in the standard gaits as walk, trot and gallop/canter. The Icelandic horse is one of many gaited breeds able to perform tölt and pace, as well as the standard gaits walk, trot and canter/gallop. The gait tölt is a four-beat symmetrical gait, with a footfall sequence where the horse always has one or two legs on the ground, without any suspension. Defined by International Federation of Icelandic horse association (FEIF) in the FIZO (2015) Rules for Icelandic Horse Breeding a good quality tölt has good speed with suppleness, clear beat, high and elegant action and big movements with long strides. A five-gaited Icelandic horse can also perform the two-beat gait pace, which unlike the two-beat trot where the horse moves diagonally and with suspension, the horse moves its legs simultaneously and lateral (Anderson et al. 2012). A good quality pace is secure, of high speed with good suspension and with clear beat, as defined by FEIF.

Prior Studies

In a previous preliminary genome wide association analysis study (GWAS) by Shrestha et al. (unpublished), they genotyped 239 Icelandic horses for 50 000 SNP markers and investigated a potential association between the SNPs and estimated breeding value (EBV) for tölt. In the study, they found a significant association with one marker on chromosome 7 and one on chromosome 13. They also did a genome wide association study (GWAS) using only Dutch Icelandic horses and were able to detect a marker on chromosome 28. A later preliminary study by Axling (unpublished) where they used 345 Icelandic horses for genotyping chromosome 13 and 348 Icelandic horses for genotyping chromosome 28, attempted to confirm the results from the previous study (Shrestha et al. unpublished), by analysing the three top markers and the association with EBV for tölt and EBV for slow tölt. Axling (unpublished), were unable to confirm the results from the study by Shrestha regarding a connection between the SNP marker on chromosome 7 and EBV. For the markers on chromosome 13 and chromosome 28 there were some significant associations found for EBVs for tölt and slow tölt. Axling (unpublished), believed that the results from the genotyping of the SNPs on chromosome 13 and 28 could implicate that these markers may be affecting the quality of the tölt.

Aim of the Study

The purpose of this study was to follow up the results from the previous preliminary studies by using phenotypic values from breeding evaluations instead of EBVs. The aim was to try to determine if there were any associations between the SNP markers on chromosome 13 and chromosome 28 and tölt scores and to investigate whether these markers affect the quality of the tölt of the Icelandic horse. The hypothesis was that there is a connection between the SNP markers on chromosome 13 and 28 and tölt. If the hypothesis is true it can have an impact on how we think about breeding as it would be possible to breed horses with certain genotypes in order to get better gaits directly affecting the price of the offspring. This would also in turn have an effect on competitions with enhanced speed, action, acceleration and quality of the gaits in other breeds as well for the Icelandic horse. Today there is much money going into breeding and sport. A good Icelandic breeding stallion sells for 30 000 € and more and it is because of the expense for a good stallion it is extremely important that the stallion not only proves himself in breeding through his offspring as a return on the initial investment, but also in competitions and shows. If the connection is proven, it will potentially have a major impact on breeding in such a way that breeders will want to breed horses with the right genotypes for tölt and slow tölt.

MATERIAL AND METHODS

For this study we genotyped 95 five-gaited Icelandic mares, which all had been judged in breeding assessments at the ages of 5-6 years and were born in Sweden. All horses that were chosen had existing hair samples at the Animal Genetics Laboratory, Swedish University of Agricultural Sciences, Uppsala, Sweden. We selected horses that had different breeding scores in tölt from their breeding evaluation test at the ages 5-6 years and that were as unrelated as possible. Mares that were closely related to each other by both sire and dame (being full siblings) were removed randomly and one of each sample was kept. Mares being half siblings by sire were kept as we did not have enough hair samples to remove all closely related horses. We wanted horses with as much variation as possible in breeding scores and pedigree to get as an accurate result as possible in this study. To signalling out witch half siblings to use, we choose at random the once with highest and/or lowest scores. We had 57 half siblings in total, and these half siblings were sired by 24 different stallions.

DNA Isolation and SNP Genotyping

A standard hair preparation method was used to isolate DNA from the hair roots. The method used was the Chelex procedure (registered trademark of Bio-Rad Laboratories, Inc., Hercules, CA). The Chelex procedure involves 7 µl of proteinase K (20 mg/mL; Merck KGaA, Darmstadt, Germany) which degrades the DNA, and a 100 ml Chelex 100 Resin (Bio-Rad Laboratories, Hercules, CA) that binds the leftover of the hair in the sample. The mix is then incubated at 56 °C during one hour and the proteinase K is

thereafter inactivated for 10 minutes at 95 °C. The samples were genotyped for two SNP markers, one on chromosome 13 (22,296,211 bp) and one on chromosome 28 (35,485,344 bp). SNP genotyping was performed with the StepOnePlus Real-Time PCR System (by Life Technologies (Thermo Fisher Scientific, Waltham, MA), using custom designed TaqMan SNP Genotyping Assays (Applied Biosystems by Life Technologies (Thermo Fisher Scientific) as has been previously described by Andersson et al. (2012) and Promerová et al. (2014).

Statistical Analysis

The statistical analyses were executed with Simple Interactive Statistical Analysis (SISA; Quantitative Skills, 2013). T-test was used for comparing the averages for tölt scores for the different genotypes and to see if there were any association between the SNP markers on chromosome 13 and 28 and tölt scores from breeding evaluations.

RESULT

Table 1 shows the genotype and allele frequencies for the SNP markers on chromosome 13 and chromosome 28. For the SNP marker on chromosome 13, the dominating allele was T with 64 % frequency and the lowest frequency was allele G with 36 %. For the SNP marker on chromosome 28, the dominating allele was A with 83 % frequency and the lowest frequency was allele G with 17 %.

Table 1. The allele and genotype frequency distributions for the SNP markers on chromosome 28 and 13

Allele and genotype frequency				
Genotypes Chromosome 28	AA	AG	GG	Allele A
Frequency	0.69 (n=66)	0.30 (n=28)	0.01 (n=1)	0.83
Genotypes Chromosome 13	GG	GT	TT	Allele T
Frequency	0.14 (n=13)	0.43 (n=41)	0.43 (n=41)	0.64

Table 2-5 shows the average, median and standard deviations, for the breeding evaluation scores of tölt and slow tölt for horses with different genotypes for the SNP markers on chromosome 28 and chromosome 13. There were no significant associations found between the SNP markers on chromosome 28 and chromosome 13 and the tölt scores. In table 2 and 3, the values for the genotype GG was eliminated thus there was only one horse that had the genotype GG and could not be used in the study.

Table 2. Average, standard deviation (SD) and median for breeding evaluation scores for slow tölt for different genotypes, and the calculated p-value of the T-test between the average scores for respective genotype on chromosome 28

	AA (n=66)	AG (n=28)	P (AA/AG)
Average	7.61	7.59	0.54
Median	7.75	7.75	
SD	0.75	0.86	

Table 3 Average, standard deviation (SD) and median for breeding evaluation scores for tölt for different genotypes, and the calculated p-value of the T-test between the average scores for respective genotype on chromosome 28

	AA (n=66)	AG (n=28)	P (AA/AG)
Average	7.86	7.77	0.79
Median	8.00	8.00	
SD	0.43	0.63	

Table 4 Average, standard deviation (SD) and median for breeding evaluation scores for slow tölt for different genotypes, and the calculated p-value of the T-test between the average scores for respective genotype on chromosome 13

	GG (n=13)	GT (n=41)	TT (n=41)	P (GG/GT)	P (GG/TT)	P (GT/TT)
Average	7.46	7.54	7.73	0.38	0.12	0.13
Median	7.50	8.00	8.00			
SD	0.75	0.86	0.70			

Table 5 Average, standard deviation (SD) and median for breeding evaluation scores for tölt for different genotypes, and the calculated p-value of the T-test between the average scores for respective genotype on chromosome 13

	GG (n=13)	GT (n=41)	TT (n=41)	P (GG/GT)	P (GG/TT)	P (GT/TT)
Average	7.69	7.82	7.90	0.21	0.10	0.22
Median	7.50	8.00	8.00			
SD	0.60	0.46	0.49			

DISCUSSION

This study demonstrated no significant associations between the SNP markers for chromosome 13 and chromosome 28 and the evaluation scores for tölt and slow tölt in the Icelandic horse. It could simply be that there is no association between these two SNPs and the tölt. However, as both preliminary studies by Shrestha et al. (unpublished), and Axling (unpublished), showed some significant associations between the SNP markers for chromosome 13 and chromosome 28, this study may not have had enough sample horses to show a significant result. However, there is a tendency for higher scores for horses with allele TT compared to horses with allele GG on chromosome 13. In their studies they genotyped over 300 horses, whiles in this study we only genotyped 95 horses. Furthermore, instead of using EBV, which they did in the studies from Shrestha et al. (unpublished) and Axling (unpublished), this study used scores from the actual evaluation test. Estimated breeding values (EBV) is calculated by a computer program, it is an estimate derived from the calculated EBV of the horse, its parents and its ancestors. The positive side of using actual breeding test scores is that horses are scored solely by their performance, gaits and natural talent during the day of the test, while the negative side is that there is a potential for human factors at play, there is the form of the horse and rider on the day of the test, the quantity and quality of the training that the horse has received prior the test and the judges perception. The advantage of using the estimated breeding values is that it is a highly specialized designed program which calculates the scores with the help of the whole pedigree of the horse, while the disadvantage is that no matter how much we calculate or how good match-making we do between horses, nature may follow a different course than expected based on estimated values. A sire and dam which both have a highly rated pedigree and have the top-scores can still give below averaged gaited offspring whiles a sire and dam with no special talent more than being loved by their owner can suddenly surprise us by giving a highly talented offspring.

Another potential and likely factor affecting the results in this study compared to the preliminary studies by Shrestha et al. (unpublished), and Axling (unpublished), is that by using actual breeding evaluation scores the variance may not be significant given that the Icelandic horses going to breeding shows are usually the ones that are anticipated to reach high scores given potentially high EBVs while the studies using EBVs used sample horses which would normally not be taken to a breeding show given the anticipated low

scores. What also could have affected the results of the previous and the current studies, which now show as a difference in the findings, besides the human error, is the fact that the two previous studies used both mares and stallions whilst in this study we only used mares. This study excluded stallions, for the very reason that a stallion can leave hundreds of offspring during one lifetime while a mare can only leave a few offspring, horse owners normally only keep stallions without castrating them if they show exceptionally high potential because of difficulties of the upkeep and there is also a higher cost of evaluating a stallion compared to the breeding assessments of mares and geldings (SIF). Because of the extra expense, the stallions chosen for the breeding evaluation tests are only the very best, which results in high breeding values. The mentioned expenses are the training leading up to the breeding show, especially for stallions which are more expensive to train, the cost of having someone showing the horse and the actual breeding show fee. In order to get as much variation in the scores for slow tölt and tölt as possible, we subsequently chose to exclude stallions from this study, as only using horses with high breeding values points would not show enough variations in the scores for slow tölt and tölt. The question thought, if we had used EBV and stallions in this study as they had done in the preliminary studies, would we have gotten a significant result?

The heritability for tölt is 0.5 and for pace 0.6 according to the study by Albertsdóttir et al. (2011). As mentioned in the studies by Andersson et al. (2012) and Kristjánsson et al. (2014) it seems that the ability to pace has a positive effect on the tölt but a negative effect on other gaits, such as gallop. As most people who buys Icelandic horses wants a horse with good tölt, they often chose a five-gaited horse, i.e. one that are homozygous for the *DMRT3* mutation. It is normally easier to get the horse to tölt and to hold the tölt with a five-gaited Icelandic horse, but it does not necessary mean that the horse have a better or clearer tölt. A four-gaited horse, which does not possess pace, is often easier to get a clear beat from, but depending on the horse and the level of the rider, it might be harder to teach the horse to tölt and to keep it. As breeding have showed us, mating two four gaiters together jeopardize the ability to tölt altogether, which can be explained with the help of the *DMRT3* mutation (CA). What could arise from mating two four gaiters (CA) is that you get a CC horse which means a three gaiter, i.e. a horse which is unable to perform the alternative gaits. Kristjánsson et al. (2014) study showed that homozygosity for the AA genotype was needed for the ability to pace and had an advantageous effect on scores for tölt, as the five gaited horses displayed better beat quality in the tölt, speed capacity and suppleness.

It would be interesting to redo this study but at a much bigger scale using a wider variety of scores and horses. If Shrestha et al. (unpublished), and Axling (unpublished), theories about the association between the SNP markers on the chromosomes 13 and 28 and their ability to affect the tölt and slow tölt are correct, it could have an impact on breeding, and in turn competition, to get horses with better quality tölt. Horses with better quality tölt will sell for more money and better tölt implicate higher scores on the competition tracks. It would also be interesting to widen the study and not only look into a correlation between scores in tölt and slow tölt and the SNP markers in the chromosomes 13 and 28. It would be interesting to make further studies on other breeds to find out if there are any significant effect on for example speed, trot, action and stride. Furthermore, using horses

from different countries could also prove to be an advantage, as you then get breeding valuation scores from different countries, even if all the countries follow the same protocol to give breeding valuation scores they will always vary a bit country from country.

CONCLUSIONS AND HYPOTHESIS TESTING

The conclusion of this study is that our hypothesis was false, as we were unable to prove that the SNP markers on chromosome 13 and chromosome 28 have an impact on slow tölt and tölt of the Icelandic horse using breeding scores. It would be interesting to redo this study, but with more horses and horses from all different countries to try to get as an accurate result as possible with more variance and later on using samples from other breeds.

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