DEVELOPMENT OF GENOMIC METHODS AND TOOLS FOR AN EQUINE MODEL

A Dissertation

Presented to the Faculty of the Graduate School

of Cornell University

In Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy in Animal Science

by

Mohammed Ali Obaid Al Abri

August 2015

DEVELOPMENT OF GENOMIC METHODS AND TOOLS FOR AN EQUINE MODEL

Mohammed Ali Al Abri, Ph.D.
Cornell University 2015

The advent of genomic analysis has identified regions of functional significance in several mammalian species. However, for horses, relatively little such work was done compared to other farm animals. The current archive of genetic variations in the horse is mostly based on the Thoroughbred mare upon which the reference sequence (EquCab2.0) was generated. Thus, more investigation of the equine genomic architecture is critical to better understand the equine genome.

Chapter 2 of this dissertation represents an analyses of next generation sequencing data of six horses from a diverse genetic background. I have utilized the most advanced techniques to identify, and annotate genetic variants including single nucleotide polymorphism, copy number variations and structural variations pertaining to these horse breeds. The analysis discovered thousands of novel SNPs and INDELs and hundreds of CNVs and SVs in each of the horses. These newly identified variants where formatted as online tracks and should provide a foundational database for future studies in horse genomics. Chapter three of the thesis discusses a genome wide association study aimed at the discovery of QTLs affecting body size variation in horses. I used the Illumina Equine SNP50 BeadChip to genotype 48 horses from diverse breeds and representing the extremes in body size in horses. Unlike most association studies, I have utilized a dominant model to identify these QTLs. The analysis revealed an association in chromosome one at the *ANKRD1* gene (involved in muscle myocytes and cardiomyocyte growth and differentiation). In chapter four, I represent the results of a genomic study in which 36 Egyptian Arabian horses were genotyped using the Illumina Equine SNP70 BeadChip. The study was conducted to elucidate the genetic background of the herd, relatedness within the herd and to estimate genomic inbreeding values. I was able to re-establish the genetic links between the horses and to confirm their Egyptian ancestry among other Arabian horse bloodlines. Genomic inbreeding values were highly correlated with the pedigree estimated ones. Altogether, our results signify the benefit of using this BeadChip technology to infer relationships within herds and ancestry of herds and to estimate inbreeding in herds lacking pedigree recording.

BIOGRAPHICAL SKETCH

Mohammed Ali Al Abri was born in Shimla, Himachal Pradesh in India. He spent the first five years of his life in Egypt and then moved to Oman. In Oman, he grew up inspired by his hard working father who always pushed him for the next big thing. He was fond of reading and learning new things and was always inquisitive. He joined the College of Agriculture at the Sultan Qaboos University in the year 2000 and graduated with a BSc in Animal Science in 2004. In the summer of 2004 he joined the Animal Science department as a faculty member. Between 2005 and 2008, he attended McGill University where he completed a master degree in dairy cattle quantitative genetics under the supervision of Roger I. Cue. He was admitted to Cornell University in 2011 to pursue his PhD in animal genetics. In 2012 he was very lucky to join Dr. Samantha Brooks equine genetics lab where he was trained in horse genetics and genomics. Mohammed also received a minor in applied statistics and completed online USDA-funded M.Sc. level modules in animal breeding. He is interested in philosophy and history and enjoys learning new programming languages.

DEDICATION

I dedicate this thesis to my mother, my father, my sweet wife Abeer and my wonderful joyful son Yazeed. I also dedicate it to my friends, Jonathan Wijtman, Paul Robillard and Tatu Lukka. This thesis is especially dedicated to my PhD supervisor Dr Samantha Brooks; without her continuous support, help and dedication it would not have been possible.

ACKNOWLEDGMENTS

I would like to thank my father who encouraged me along the way and taught me to aim high, my mother who taught me many valuable life lessons and always had faith in me. A sincere thank you goes to my wife Abeer. Without the comfort and support she provided me; accomplishing this wouldn't have been an achievable task. I would also like to thank my amazing and supportive adviser Dr. Samantha Brooks. She was always patient, understanding, and flexible and she enthusiastically supervised my research. I am very grateful for Dr. Roger Ian Cue, whose supervision and teaching during my MSc prepared me for much of the programming skills and animal genetics knowledge I needed in my PhD. My gratitude goes to Dr. Albano Beja Pereira (University of Porto) for all the valuable and fruitful discussions on my projects. I would like to also thank Didier Chételat for his help and encouragement in my statistics classes and to Kaixiong Ye for his inspiring thoughts and collaboration in the classes we took together. From our lab, I would like to thank Heather Holl, Chris Posbergh and Ann Staiger for always been there when I needed help. Special thanks as well go to Dr. Ron Lewis of Virginia Tech for admitting me to the online graduate courses in animal breeding and genetics. These courses have been wonderful and very beneficial in increasing my understanding of animal breeding. I would like to additionally thank my committee members, Dr. Jason Mezey who was always there for me whenever I needed advices throughout my PhD, Dr. James Booth and Dr. Adam Boyko who provided me the support and guidance related to my project and studies in general.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

INTRODUCTION

Producing a whole-genome sequence of an organism is a fundamental step towards understanding its genetic architecture, identifying the sequence and special pattern of expressed genes, and characterizing genomic variations at the base pair level. It also helps understanding the demographic and evolutionary history of various breeds/varieties and to catalog them according to their significance. Therefore, the availability of genomic sequence data is essential for the characterization and conservation work carried out on both captive and wild horse populations. The works documented in the subsequent chapters discuss the evolution of sequencing and genotyping technologies and the related statistical techniques used to leverage their capabilities. Additionally, the novel genomic techniques and their potential in improving our understanding of the horse genome, genes and diversity are discussed.

DNA sequencing technology began with the development of the Sanger sequencing (Sanger *et al.* 1977) in the late 1970s. This sequencing method uses chain termination of dideoxynucleotides followed by capillary electrophoresis size separation of fragments and finally detection of nucleotide bases using florescence dyes. Gradual development of the Sanger sequencing method has improves sequence length up to approximately 1000 bp with per-base accuracies as high as 99.99% (Wang *et al.* 2012). Excellent accuracy and long reads quickly established the Sanger sequencing method as a standard in the industry. Dominating the DNA sequencing market for nearly two decades, it lead to the completion of a number of high quality whole genome sequences including that of the humans (Metzker 2010). However, the cost and long run time for the Sanger sequencing technology made it prohibitive to utilize for project with limited funding.

The demand for cheaper and faster sequencing methods resulted in the development of what is now known as Next Generation Sequencing (NGS) technologies in the mid-2000. NGS technology can be generally divided into 2nd and 3rd generations sequencing technologies (Glenn 2011). Most 2nd generation sequencing platforms, such as the 454/Roche, Solexa/Illumina, and the SOLiD platform (Applied Biosystems), follow the cyclic array sequencing approach (Shendure *et al.* 2008). In principle, the approach is inspired by the shotgun sequencing which was first implemented by the Human Genome Project (Zhang *et al.* 2011) in order to refine the human genome. It basically involves shearing the genomic DNA, ligating adapters unto it, amplifying a library of millions of similar DNA fragments through polymerase chain reaction (PCR) and then sequencing using an approach unique to each platform.

On the other, hand 3rd generation sequencing platforms sequence individual DNA molecules directly without the need for amplification. It is generally faster in both sample preparation and run time required. Additionally, the sequences are typically longer than 2nd generation sequencing, for example the PacBio RS system can reach up to tens of kilobases (van Dijk *et al.* 2014). This made it the method of choice for improving current genome assemblies especially in highly repetitive areas of the genome. However, high error rates and relatively low throughput are still limiting factors for 3rd generation sequencing technologies (van Dijk *et al.* 2014).

Compared to Sanger sequencing, NGS technologies are much faster and cheaper and have outperformed Sanger technology by a factor of 100-1000 in terms of daily yield (Kircher & Kelso 2010). Using Sanger technology, the estimated cost of the human genome project which took approximately 13 years to complete was about 3 billion dollars (Hayden 2014). On the other hand, re-sequencing a human genome using current NGS platforms typically costs less than $10,000 and takes about a week. However, NGS technologies come with their own disadvantages, notably short read length and higher error rate compared to Sanger sequencing.

Illumina technology is one of the most widely used sequencing technologies and currently outperforms other NGS in the number and percentage of error-free reads (Glenn 2011). The Illimina Hi-Seq 2000 Genome Analyzer released in 2010 is able to produce > 200 giga basepair (Gbp) of 2 × 100 base reads per run, with a raw accuracy of the bases higher than 99.5% (Zhang *et al.* 2011). The extremely high throughput produced by this machine translates to a higher depth of coverage across the genome, providing more certainty to the genomic variants called using this technology. In early 2014, Illumina announced the release of the HiSeq X Ten (a collection of ten HiSeq X sequencers) which is capable of producing 1.8 Tera base pairs of sequence per run at a cost of only $1000 dollars.

Illumina paired end reads (reads of both ends of a DNA fragment) are generated by sequencing from each end of DNA template, leaving out the middle portion of the template, in a process known as bridge amplification. These reads can be very useful for detecting genomic variants and chromosomal rearrangements such as deletions, insertion and inversion. However, the same reads can be also used to detect copy number (CNVs) as well as single nucleotide polymorphisms (SNPs). In principle, there are two ways to analyze these reads. The first one is to generate a *de novo* assembly of the genome followed by variants discovery using specialized software. *De novo* assembly can be either guided or unguided by the reference genome of the organism and is usually computationally demanding, especially when utilizing the relatively short 100 bp paired end reads. The second method, guided assembly, maps the reads to the reference and then calls variants.

The assembly of the current equine reference (EquCab 2) was completed in 2009 by the Broad Institute of MIT and Harvard (Wade *et al.* 2009) after sequencing the DNA of an inbred Thoroughbred Mare named Twilight using primarily the Sanger method off sequencing. The genome is considered of a high quality since its depth of coverage is 6.8x and > 95% of its assembled sequences were anchored to the equine chromosomes. Also, the N50 contig size of the genome is 112-kb and it has a 46-Mb N50 scaffold size. With 53% of horse genome showing similarity to a single human chromosome, it is considered closer in structure to the human genome than many mammalian genomes including the dog (which has just 29% homology to humans) (Wade *et al.* 2009). Therefore, it is not surprising to know that the horse shares about 90 genetic disorders that may serve as models for human disorders (Wade *et al.* 2009). However, in spite of the fact that the horse genome is considered high quality, about 5 % of the sequences were not anchored to chromosomes and now make up what is known as chromosome unknown (chrUn) in the current, EquCab2.0, assembly. Utilization of the current genome assembly is also hindered by a lack of horse specific annotation as most genes are based on computational predictions from other species. Realizing the potential for improvement of EquCab2, a team of equine geneticists have recently begun work on the third version of the assembly and has so far generated 40X of Illumina paired end and mate pair sequence data from Twilight to complement the existing Sanger sequencing data used to create EquCab2 (Kalbfleisch T 2015).

In addition to the lack of gene annotations, the archive of genetic variations in the horse includes variants from only a handful of breeds. The current database includes variants that were primarily identified by the Horse Genome Project , and re-sequencing studies of the Quarter horse (Doan *et al.* 2012) and the Marwari horse (Jun *et al.* 2014). The total number of non-redundant SNPs yielded by these studies is approximately 6 million SNPs. Nevertheless, there are approximately 400 distinct breeds of horses each with a unique physical characteristics (Hendricks 1995). Therefore, to capture the breadth of existing functional variation in the horse, additional horse breeds need to be sequenced. The genetic variation that would be discovered in these breeds is a valuable addition to the variants currently available and aid investigating the relationships among them in a comparative genomics framework. Additionally, breed specific variants shed more light on the genetic uniqueness of each of the breeds and its selection and domestication history.

In chapter one, we utilized Illumina NGS technology to fully re-sequence six horses from diverse breeds using the paired end sequencing approach. After filtering and mapping the reads to EquCab2 we used cutting edge variant discovery tools in order to discover genetic variation unique to each horse. These newly discovered variants will enrich the current genetic variant archive of the horse. They are now available to the horse genomics community and can be loaded easily into genome viewing web interfaces such as University of California Santa Cruz (UCSC) genome browser.

Obtaining a complete archive of genetic variants is imperative since variation at the sequence level is eventually manifested as phenotypic variation between individuals. Genetic mapping, or association, is aimed at the detection and localization of genetic variation underlying phenotypic variation. The idea of genetic association studies is not new. In the 1950s, a study suggested the association between blood-group antigens and peptic ulceration (Aird *et al.* 1954). In the 1980s, apolipoprotein E locus (APOE) was found associated with variations in the onset and risk of Alzheimer's disease (Strittmatter & Roses 1996). In general, most early genetic mapping studies used only a handful of genetic markers. Recently however, the developments of SNP genotyping arrays for humans as well as the majority of economically important livestock species have revolutionized genetic mapping. These arrays provide genotypic information on thousands to millions of SNPs across the genome depending on species of interest. Genome wide association studies (GWAS) are a form of genetic mapping that involves utilizing these arrays in genotyping SNPs of many individuals to find genetic variations associated with the phenotype or phenotypes of interest. These studies have detected a number of loci involved in human phenotypes such as height (Li *et al.* 2010), breast cancer (Easton *et al.* 2007) and schizophrenia (Lencz *et al.* 2013). In horses, GWAS has also been successful in detecting loci linked to height (Makvandi-Nejad *et al.* 2012), Guttural Pouch Tympany (Metzger *et al.* 2012) and Lavender Foal Syndrome (Brooks *et al.* 2010a).

However, the systematic sharing of ancestry in cases and controls can create allele frequency differences between them leading to spurious associations or false positives unrelated to the outcome of interest (Hoffman *et al.* 2014). This systematic sharing of ancestry is commonly called population structure. Early methods suggested to help account for the population structure in GWAS beginning with genomic control (GC) (Devlin & Roeder 1999) and later principle component analysis (PCA) (Price *et al.* 2006). However, GC correction may over or under correct certain SNPs depending on their ancestry. On the other hand, if the population structure is the result of several discrete subpopulations, PCA analysis will not be able adjust for it since it uses eigenvectors as continuous covariates (Liu *et al.* 2013). Additionally, these methods do not always account for the relatedness (kinship) between individuals. In recent years, many studies have suggested using linear mixed model (LMM) to correct for population structure and kinship simultaneously (Hoffman 2013). Mixed models equations (MME) were first suggested by Henderson in 1949 (Henderson 1949) but were only formalized in 1963 (Henderson 1963). Since then, they have been used successfully up to this day in genetic evaluations to predict the genetic merit of animals and in genetic evaluations of dairy cattle, and soon after, beef cattle in the US. Predictions from MME are known as Best Linear Unbiased Predictions or BLUP, a term first coined by Goldberger (Goldberger 1964). Typically, mixed models account for relatedness by fitting the Wright's numerator relationship matrix (or the A matrix) in the as a random effect in the mixed models equations (MME). The relationship matrix can nowadays be estimated from genotypic data and would then be called the genomic relationship matrix or the G matrix.

When applying mixed models to GWAS, the G matrix can be included in the random part of the mixed model GWAS which is then defined as follows:

$$y = X\beta + Zu + e$$

Where y is an n × 1 vector of phenotypes, X is an n × q matrix of fixed effects including mean, the SNPs being tested, in addition to other confounding variables such as age or gender. β is a q × 1 vector of fixed effects coefficients vector. Z is an incidence matrix that maps the phenotype to the corresponding breed or strain. u is the random effect with Var (u) = σ_g^2 K, where K is a $t \times t$ genomic relationship matrix and $e \sim N(0, I\sigma_e^2)$ is the residual effect. The phenotypic variance covariance matrix is given by $V = \sigma_g^2 ZKZ' + \sigma_e^2 I$.

In chapter two, we fitted a G matrix in our GWAS using the software EMMA (Kang *et al.* 2008). EMMA uses a simple method to estimate the G matrix that guarantees positive semidefiniteness in GWAS. Additionally, the global restricted maximum likelihood (REML) solutions for variance components are attained using a Newton-Raphson search algorithm which is guaranteed to converge as long as the kinship is positive semidenfinite (Kang *et al.* 2008).

By fitting relationships between individuals as random effects in our GWAS, we were able to account for the cryptic relatedness between individuals and account for the population structure correctly. Using the same method in an additive model framework, Makvandi-Nejad *et al.* (2012) discovered four loci that account for more than 80% of the variation in horse body size. Using a dominant model, we detected a locus in chromosome one that was highly associated with height variation in horses. We also successfully accounted for the existing cryptic relatedness between the horses used in the study. Our finding was later confirmed using a PCR and Restricted Fragment Length Polymorphism (RFLP) test in an independent set of American Minature and Falabela horses.

Typically, mixed models GWAS presume that the trait follows an infinitesimal genetic architecture i.e all SNPs are assumed to contribute equally to the variation in phenotype. However, for dichotomous traits such as health disorders, that assumption may not be true as these traits could be affected by few major genes as in Lavender Foal Syndrome (Brooks *et al.* 2010a). Modeling the genetic architecture using a noninfinitesimal model so that most SNPs have a small effect while others have major effect can therefore increase the GWAS power for such traits (Tucker *et al.* 2014). Bayesian models allow for the flexibility of specifying the genetic architecture of the trait so that, depending on prior knowledge of its nature, its genetic architecture can be modeled more accurately.

Akin to mixed models, Bayesian models in animal breeding were developed to be used primarily for genetic evaluations. However, many researchers started using them for GWAS due to their flexibility in modeling the genetic variance attributed to SNPs. A fundamental difference between Bayesian and Mixed models GWAS is that Bayesian models fits all the SNPs as random effects in the model simultaneously where as in Mixed models, SNPs are fitted individually and their effects are estimated separately from one another. Additionally, in the mixed models framework, each SNP is assigned a p-value indicating the magnitude of its association with the phenotype where as in Bayesian models GWAS, the SNP effects are calculated as percentage of variance explained inferred from posterior distributions. The general form of the Bayesian statistical model is:

$$y = X\beta + \sum_{i=1}^{k} z_{ik}\,\alpha_k + e$$

where *y* is the vector of phenotypes, *X* is the fixed effects incidence matrix, β is the fixed effects solutions vector, *K* is the number of SNPs, z_{ik} is the value of the SNP *k* (*k*= 0, 1, or 2) *pertaining* *to individual i* . α_k is the substitution effect of SNP *k, with* α_k sampled from N(0, $\sigma^2_{\alpha k}$) with a probability of (1−π) and α_k=0 with probability π, where π is the fraction of SNPs with no effect. When π =0.5 half of the SNPs will be drown from a distribution with 0 effects and the other half from N (0, $\sigma^2_{\alpha k}$). $\sigma^2_{\alpha k}$ has a scaled inverse chi square distribution with 4 degrees of freedom ($v_\alpha =$ 4) and a scale parameter $S^2_\alpha = \frac{\sigma^2_g(v_\alpha - 2)}{(1-\pi)\sum_{k=1}^{k} 2p_k(1-p_k)v_\alpha}$ where p_k is the allele frequency and σ^2_g is the additive genetic variance inferred from the markers. $e \sim N(0, I\sigma^2_e)$ is the residual effect. The variance explained by the SNP *is usually* estimated using the Monte-Carlo means or medians of the posterior distribution computed by a Gibbs sampling. These Bayesian models or have been successful in mapping threshold traits in livestock species such as calving ease in beef cattle (Peters *et al.* 2013) and continuous traits such as body composition in pigs (Fan *et al.* 2011). Another application of the genome-wide SNP genotypes provided by high-throughput arrays is the calculation of genomic inbreeding values. Calculating inbreeding and relationships between individuals is another advantageous use of these genomic data especially in situations where pedigree information is not available or is imprecise. The classical measure of inbreeding as first proposed by Wright (1922) was calculated using pedigree information. This measure was meant to estimate the proportion of the genome that is identical by descent (IBD) and homozygous. This statistic, termed “F”, was defined as the inbreeding coefficient and is equal to one half the additive relationships between parents of an individual. Then the path method was introduced (Wright 1934) in and improved the calculation of Wright’s inbreeding coefficient. Later, a recursive method to calculate relationships and inbreeding coefficient was introduced and greatly accelerated the calculation of inbreeding coefficients in large pedigrees (Emik & Terrill 1949). The negative impact of mating individuals whose parents are related, i.e inbred animals, has long been recognized by biologists (Darwin.C 1868). Compared with outbred populations, inbred populations have a higher prevalence of recessive genetic disorders (Modell & Darr 2002). Therefore, accurate estimates of inbreeding are essential in mating decisions and assessment of herd genetic diversity. In the field of animal breeding, algorithms were developed to directly control long term inbreeding while maximizing genetic gain in what is known as optimal contribution selection (Meuwissen 1997). However, pedigree based inbreeding estimates are only as good as the pedigree they were estimated from. The depth and quality of the pedigree can largely impact them and imprecise pedigrees can largely compromise their value. Also, because they consider only the additive alleles (estimated shared loci) from parent to offspring, they do not account for mendelian sampling variation between half sibs. With the development of genotyping arrays for most agriculturally important animal and species, genome-wide inbreeding values for an individual can now be estimated empirically from levels of genomic homozygosity. Simulation results have shown that genomic calculations of inbreeding and relationships are closer to the true values than those estimated from pedigrees (Keller *et al.* 2011). This could be explained by the fact that they reflect mendelian sampling which pedigree inbreeding calculations cannot directly observe (Hill & Weir 2011). The benefit of such genomic inbreeding values was demonstrated previously in the Thoroughbred horse (Binns *et al.* 2012) and more recently in a variety of other breeds (Petersen *et al.* 2013). Within the Arabian horse population the impacts of popular sires could increase inbreeding by reducing the overall pool of mates. Therefore, obtaining accurate measures of inbreeding is imperative in order to monitor genomic diversity within breeding programs. In chapter three, we use genotypic data on a herd of 36 German Arabian horse herd and estimate various measures of inbreeding and also reconstruct genetic relationships between individuals in the herd.

The tools and techniques discussed above have certainly yielded valuable findings in each of the corresponding projects. We demonstrated the utility of those tools in the discovery of novel genetic variants, assessing the diversity of horses and enriching the current catalog of the horse genetic variation. Our findings can be utilized in improving our understanding of the horse biology and provide a starting point for future investigations.

REFERENCES

Aird I., Bentall H.H., Mehigan J.A. & Roberts J.A.F. (1954) The blood groups in relation to peptic ulceration and carcinoma of colon, rectum, breast, and bronchus; an association between the ABO groups and peptic ulceration. *British medical journal* **2**, 315-21.

Binns M.M., Boehler D.a., Bailey E., Lear T.L., Cardwell J.M. & Lambert D.H. (2012) Inbreeding in the Thoroughbred horse. *Animal genetics* **43**, 340-2.

Brooks S.A., Gabreski N., Miller D., Brisbin A., Brown H.E., Streeter C., Mezey J., Cook D. & Antczak D.F. (2010) Whole-genome SNP association in the horse: identification of a deletion in myosin Va responsible for Lavender Foal Syndrome. *PLoS genetics* **6**, e1000909-e.

Darwin.C (1868) *The Variation of Animals and Plants Under Domestication*, Murray:London.

Devlin B. & Roeder K. (1999) Genomic control for association studies. *Biometrics* **55**, 997-1004.

Doan R., Cohen N.D., Sawyer J., Ghaffari N., Johnson C.D. & Dindot S.V. (2012) Whole-genome sequencing and genetic variant analysis of a Quarter Horse mare. *BMC Genomics* **13**, 78-.

Easton D.F., Pooley K.A., Dunning A.M., Pharoah P.D.P., Thompson D., Ballinger D.G., Struewing J.P., Morrison J., Field H., Luben R., Wareham N., Ahmed S., Healey C.S., Bowman R., Meyer K.B., Haiman C.A., Kolonel L.K., Henderson B.E., Le Marchand L., Brennan P., Sangrajrang S., Gaborieau V., Odefrey F., Shen C.-Y., Wu P.-E., Wang H.-C., Eccles D., Evans D.G., Peto J., Fletcher O., Johnson N., Seal S., Stratton M.R., Rahman N., Chenevix-Trench G., Bojesen S.E., Nordestgaard B.G., Axelsson C.K., Garcia-Closas M., Brinton L., Chanock S., Lissowska J., Peplonska B., Nevanlinna H., Fagerholm R., Eerola H., Kang D., Yoo K.-Y., Noh D.-Y., Ahn S.-H., Hunter D.J., Hankinson S.E., Cox D.G., Hall P., Wedren S., Liu J., Low Y.-L., Bogdanova N., Schürmann P., Dörk T., Tollenaar R.A.E.M., Jacobi C.E., Devilee P., Klijn J.G.M., Sigurdson A.J., Doody M.M., Alexander B.H., Zhang J., Cox A., Brock I.W., MacPherson G., Reed M.W.R., Couch F.J., Goode E.L., Olson J.E., Meijers-Heijboer H., van den Ouweland A., Uitterlinden A., Rivadeneira

F., Milne R.L., Ribas G., Gonzalez-Neira A., Benitez J., Hopper J.L., McCredie M., Southey M., Giles G.G., Schroen C., Justenhoven C., Brauch H., Hamann U., Ko Y.-D., Spurdle A.B., Beesley J., Chen X., Mannermaa A., Kosma V.-M., Kataja V., Hartikainen J., Day N.E., Cox D.R. & Ponder B.A.J. (2007) Genome-wide association study identifies novel breast cancer susceptibility loci. *Nature* **447**, 1087-93.

Emik L.O. & Terrill C.E. (1949) Systematic Procedures for Calculating Inbreeding Coefficients. *J. Hered.* **40**, 51-5.

Fan B., Onteru S.K., Du Z.-Q., Garrick D.J., Stalder K.J. & Rothschild M.F. (2011) Genome-wide association study identifies Loci for body composition and structural soundness traits in pigs. *Plos One* **6**, e14726-e.

Glenn T.C. (2011) Field guide to next-generation DNA sequencers. *Molecular ecology resources* **11**, 759-69.

Goldberger A.S. (1964) *Econometric Theory*. John Wiley & Sons, Inc, New York.

Hayden E.C. (2014) Technology: The $1,000 genome. *Nature* **507**, 294-5.

Henderson C.R. (1949) Estimation of changes in herd environment. *J. Dairy Sci.*, 32:706 (Abstr.)-32: (Abstr.).

Henderson C.R. (1963) Selection index and expected genetic advance. *NAS-NRC* **982**, p.141-63.

Hendricks B. (1995) International Encyclopedia of Horse Breeds. (ed. by Press. UO).

Hill W.G. & Weir B.S. (2011) Variation in actual relationship as a consequence of Mendelian sampling and linkage. *Genet Res (Camb)* **93**, 47-64.

Hoffman G.E. (2013) Correcting for Population Structure and Kinship Using the Linear Mixed Model: Theory and Extensions. *Plos One* **8**, e75707-e.

Hoffman G.E., Mezey J.G. & Schadt E.E. (2014) lrgpr: interactive linear mixed model analysis of genome-wide association studies with composite hypothesis testing and regression diagnostics in R. *Bioinformatics (Oxford, England)* **30**, 3134-5.

Jun J., Cho Y., Hu H., Kim H.-M., Jho S., Gadhvi P., Park K., Lim J., Paek W., Han K., Manica A., Edwards J.S. & Bhak J. (2014) Whole genome sequence and analysis of the Marwari horse breed and its genetic origin. *BMC Genomics* **15**, S4-S.

Kalbfleisch T R.-M.J.O.L.M.J.N. (2015) Work Toward EquCab3: A New Reference Genome Sequence for the Domestic Horse. Plant and Animal Genome XXII. January 12th, San Diego, California, USA.

Kang H.M., Zaitlen N.a., Wade C.M., Kirby A., Heckerman D., Daly M.J. & Eskin E. (2008) Efficient control of population structure in model organism association mapping. *Genetics* **178**, 1709-23.

Keller M.C., Visscher P.M. & Goddard M.E. (2011) Quantification of inbreeding due to distant ancestors and its detection using dense single nucleotide polymorphism data. *Genetics* **189**, 237-49.

Kircher M. & Kelso J. (2010) High-throughput DNA sequencing--concepts and limitations. *BioEssays : news and reviews in molecular, cellular and developmental biology* **32**, 524-36.

Lencz T., Guha S., Liu C., Rosenfeld J., Mukherjee S., DeRosse P., John M., Cheng L., Zhang C., Badner J.A., Ikeda M., Iwata N., Cichon S., Rietschel M., Nöthen M.M., Cheng A.T.A., Hodgkinson C., Yuan Q., Kane J.M., Lee A.T., Pisanté A., Gregersen P.K., Pe'er I., Malhotra A.K., Goldman D. & Darvasi A. (2013) Genome-wide association study implicates NDST3 in schizophrenia and bipolar disorder. *Nature communications* **4**, 2739-.

Liu L., Zhang D., Liu H. & Arendt C. (2013) Robust methods for population stratification in genome wide association studies. *BMC bioinformatics* **14**, 132-.

Makvandi-Nejad S., Hoffman G.E., Allen J.J., Chu E., Gu E., Chandler A.M., Loredo A.I., Bellone R.R., Mezey J.G., Brooks S.a. & Sutter N.B. (2012) Four loci explain 83% of size variation in the horse. *Plos One* **7**, e39929-e.

Metzger J., Ohnesorge B. & Distl O. (2012) Genome-wide linkage and association analysis identifies major gene loci for guttural pouch tympany in Arabian and German warmblood horses. *Plos One* **7**, e41640-e.

Metzker M.L. (2010) Sequencing technologies - the next generation. *Nature reviews. Genetics* **11**, 31-46.

Meuwissen T.H. (1997) Maximizing the response of selection with a predefined rate of inbreeding. *Journal of animal science* **75**, 934-40.

Modell B. & Darr A. (2002) Science and society: genetic counselling and customary consanguineous marriage. *Nature reviews. Genetics* **3**, 225-9.

Peters S.O., Kizilkaya K., Garrick D.J., Fernando R.L., Reecy J.M., Weaber R.L., Silver G.A. & Thomas M.G. (2013) Heritability and Bayesian genome-wide association study of first service conception and pregnancy in Brangus heifers. *Journal of animal science* **91**, 605-12.

Petersen J.L., Mickelson J.R., Cothran E.G., Andersson L.S., Axelsson J., Bailey E., Bannasch D., Binns M.M., Borges A.S., Brama P., Machado A.D., Distl O., Felicetti M., Fox-Clipsham L., Graves K.T., Guerin G., Haase B., Hasegawa T., Hemmann K., Hill E.W., Leeb T., Lindgren G., Lohi H., Lopes M.S., McGivney B.A., Mikko S., Orr N., Penedo M.C.T., Piercy R.J., Raekallio M., Rieder S., Roed K.H., Silvestrelli M., Swinburne J., Tozaki T., Vaudin M., Wade C.M. & McCue M.E. (2013) Genetic Diversity in the Modern Horse Illustrated from Genome-Wide SNP Data. *Plos One* **8**.

Price A.L., Patterson N.J., Plenge R.M., Weinblatt M.E., Shadick N.A. & Reich D. (2006) Principal components analysis corrects for stratification in genome-wide association studies. *Nature Genetics* **38**, 904-9.

Sanger F., Nicklen S. & Coulson A.R. (1977) DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences of the United States of America* **74**, 5463-7.

Shendure J.A., Porreca G.J. & Church G.M. (2008) Overview of DNA sequencing strategies. *Current protocols in molecular biology / edited by Frederick M. Ausubel ... [et al.]* **Chapter 7**, Unit 7.1-Unit 7.1.

Strittmatter W.J. & Roses A.D. (1996) Apolipoprotein E and Alzheimer's disease. *Annual review of neuroscience* **19**, 53-77.

Tucker G., Price A.L. & Berger B. (2014) Improving the power of GWAS and avoiding confounding from population stratification with PC-Select. *Genetics* **197**, 1045-9.

van Dijk E.L., Auger H., Jaszczyszyn Y. & Thermes C. (2014) Ten years of next-generation sequencing technology. *Trends in Genetics* **30**, 418-26.

Wade C.M., Giulotto E., Sigurdsson S., Zoli M., Gnerre S., Imsland F., Lear T.L., Adelson D.L., Bailey E., Bellone R.R., Blöcker H., Distl O., Edgar R.C., Garber M., Leeb T., Mauceli E., MacLeod J.N., Penedo M.C.T., Raison J.M., Sharpe T., Vogel J., Andersson L., Antczak D.F., Biagi T., Binns M.M., Chowdhary B.P., Coleman S.J., Della Valle G., Fryc S., Guérin G., Hasegawa T., Hill E.W., Jurka J., Kiialainen a., Lindgren G., Liu J., Magnani E., Mickelson J.R., Murray J., Nergadze S.G., Onofrio R., Pedroni S., Piras M.F., Raudsepp T., Rocchi M., Røed K.H., Ryder O.a., Searle S., Skow L., Swinburne J.E., Syvänen a.C., Tozaki T., Valberg S.J., Vaudin M., White J.R., Zody M.C., Lander E.S. & Lindblad-Toh K. (2009) Genome sequence, comparative analysis, and population genetics of the domestic horse. *Science (New York, N.Y.)* **326**, 865-7.

Wang X.V., Blades N., Ding J., Sultana R. & Parmigiani G. (2012) Estimation of sequencing error rates in short reads. *BMC bioinformatics* **13**, 185-.

Wright S. (1922) Coefficients of inbreeding and relationship. *Am Nat* **56**, 330-8.

Wright S. (1949) THE GENETICAL STRUCTURE OF POPULATIONS. *Annals of Eugenics* **15**, 323-54.

Yang J., Benyamin B., McEvoy B.P., Gordon S., Henders A.K., Nyholt D.R., Madden P.A., Heath A.C., Martin N.G., Montgomery G.W., Goddard M.E. & Visscher P.M. (2010) Common SNPs explain a large proportion of the heritability for human height. *Nature Genetics* **42**, 565-9.

Zhang J., Chiodini R., Badr A. & Zhang G.F. (2011) The impact of next-generation sequencing on genomics. *Journal of Genetics and Genomics* **38**, 95-109.

WHOLE GENOME DETECTION OF SEQUENCE AND STRUCTURAL POLYMORPHISM IN SIX DIVERSE HORSES REVEALS LOCI UNDER SELECTION FOR BODY SIZE AND ATHLETICISM

Mohammed A Al Abri[1,2], Sara E Kalla[3], Douglas F. Antczak[4], Nate Sutter[5] and Samantha Brooks[6]

[1]Department of Animal Science, Cornell University, Ithaca, NY 14853, USA

[2]Department of Animal and Veterinary Sciences,College of Agriculture and Marine Sciences, Sultan Qaboos University, PO box 34 Al Khod, Postal Code 123, Muscat, Oman

[3]Department of Clinical Sciences, College of Veterinary Medicine, Cornell University, Ithaca, NY 14853, USA

[4]Baker Institute for Animal Health, Cornell University, Ithaca, NY 14853, USA

[5]La Sierra University, Department of Biology, 4500 Riverwalk Parkway, Riverside, CA 92515

[6]Department of Animal Science, University of Florida, PO Box 110910,Gainesville, FL 32611

*Corresponding author

*Now Department of Animal Sciences, University of Florida, Gainesville, FL 32611, USA

ABSTRACT

Completed in 2009, the reference genome assembly of the domesticated horse (EquCab 2.0) produced the majority of publically available annotations of genetic variations in this species. Following that effort a few other projects have focused on variant discovery, but only in a particular breed or two. In this project we aim to identify and annotate single nucleotide polymorphisms (SNPs), insertions and deletions (INDELs), copy number variations (CNVs) and structural variations (SVs) in the genomes six horses of diverse genetic background using next generation sequencing. We used paired-end Illumina sequencing to interrogate the genomes of an Arabian, a Percheron, an American Miniature, Mangalarga Marchador (Brazil), Native Mongolian Chakouyi, and a Tennessee Walking Horse to an average sequence coverage of 10x to 24x. Employing the GATK haplotype caller as well as the existing dbSNP variants as priors, we utilized an iterative approach for variant discovery that resulted in the identification of 8,128,658 SNPs and 830,370 INDELs. We also discovered an average of 924 CNVs and 5336 SVs regions in each of the horses and functionally annotated these features using ENSEMBL gene models. To facilitate accessibility to our findings, we formatted all the discovered variants into user friendly tracks, currently hosted in public databases. Genome-wide diversity (π) revealed regions involved in skeletal development in the Percheron horse including *MYO3B*, *HOXD12*, and *HOXD1* on ECA 18 and *ANKRD1* (ECA 1). Our SV analysis also detected a putatively functional duplication in *ZFAT* gene (ECA 9) unique to the American Miniature horse and an inverted duplication unique to the Percheron horse in *HMGA1*. Our CNV analysis detected a copy number gain in a gene cluster that includes the latherin gene (LATH) that could be the result of an evolutionary selection for heat endurance and athleticism in the horse.

INTRODUCTION

Understanding genetic variation is an important theme in modern biology and population genetics. Technological advances in genomics in recent years greatly benefitted livestock genomics in that they allow examination of genetic variation at an unprecedented scale and resolution. Cataloging that variation lays the ground for dissecting the complex genetic architecture of different traits which has a much anticipated application in livestock health, welfare, physiology and production traits (Womack 2005; Daetwyler *et al.* 2014). It also improves inference of ancient demographic and evolutionary histories and the mechanisms underlying the adaptability of the species (Orlando *et al.* 2013). In addition, cross-species comparison of genetic variation allows a better understanding of the mammalian genome through comparative genomic studies (Thomas *et al.* 2003).

Domesticated approximately 5,500 years ago, horses are one of the oldest livestock species to be domesticated and were historically used for transportation, trade warfare and as draught animals (Schubert *et al.* 2014). Throughout domestication, horses were selected for a range of physical and behaviorally desirable traits resulting in the formation of more than 400 horse breeds (Warmuth *et al.* 2015) . A study comparing ancient to domesticated horses genomes revealed 125 potential domestication target genes that have undergone positive selection (Schubert *et al.* 2014). Advantageously, the equid species possess a particularly old and diverse fossil record, aiding not only in characterizing their demographic history but also ancient human movement and migration (Orlando *et al.* 2013; Schubert *et al.* 2014; Warmuth *et al.* 2015). Nevertheless, compared to other livestock species, relatively few studies have focused on the discovery of the standing genetic variation within different horse breeds (Doan *et al.* 2012).

Currently, there are only about 5,572,537 SNPs (www.ncbi.nlm.nih.gov/projects/SNP/ [build 144]) cataloged in the database for genetic variation (dbSNP) for the horse. The majority of these SNPs were discovered in only two studies, one of which was the genome assembly project (Wade *et al.* 2009) and the other a genome re-sequencing study limited to a single American Quarter Horse (Doan *et al.* 2012). Therefore, additional investigation of the equine genomic architecture is critical for a better understanding of the equine genome *per se*, and also for expanded comparisons of variation across diverse mammalian species. Furthermore, the equine industry itself provides an eager opportunity to apply genomic discoveries towards improvements in the health and well-being of this valuable livestock species.

Our objective was to enrich the current collection of genetic variants in the horse, and to provide some functional prediction for these newly identified variants, including single nucleotide polymorphisms (SNPs), copy number variations (CNVs) and structural variations (SV). As a result of its remarkably high sequencing throughput, Next Generation Sequencing (NGS) provides access to the large collection of the existing genetic variation in the genome. Therefore, we used Illumina paired-end NGS technology to sequence the genomes of six horses belonging to six diverse horse breeds. Namely, the chosen individuals were two females, an American Miniature and a Percheron, as well as four males, an Arabian, a Mangalarga Marchador, a Native Mongolian Chakouyi, and a Tennessee Walking Horse. Aside from an extreme contrast in body size, these horses were also selected to perform distinct tasks and, hence, each has developed its own unique adaptive physiology. After applying rigorous filtration criteria to the read qualities, we detected and annotated SNPs, INDELs, CNVs and SVs in the six horses. These genetic variants will be useful for many future research projects. They are now publically available in dbSNP, dbVAR as well as in the National Animal Genome Research Program (NAGRP) VCF Data Repository.

CNVs and SVs are often difficult to access in public databases, therefore we have processed these novel variants into user-friendly tracks available for download at http://www.animalgenome.org/repository/horse.

MATERIALS AND METHODS

DNA Collection and Whole Genome Sequencing

DNA was extracted from either blood using Puregene whlole-blood extraction kit (Qiagen INc., Valencia, CA, USA) or hair samples using previously published methods (Locke *et al.* 2002). Paired-end sequencing was performed at the Biotechnology Resource Center, Cornell University. For the construction of sequencing libraries, genomic DNA was sheared using a Covaris acoustic sonicator (Covaris, Woburn MA) and converted to Illumina sequencing libraries by blunt end-repair of the sheared DNA fragments, adenylation, ligation with paired-end adaptors, and enriched by PCR according to the manufacturer's protocol (Illumina, San Diego CA). The size of the sequencing library was estimated by capillary electrophoresis using a Fragment Analyzer (AATI, Ames IA) and Qubit quantification (Life Technologies, Carlsbad CA). Cluster generation and paired-end sequencing on Illumina HiSeq instruments were performed according to the manufacturer's protocols (Illumina, San Diego) at the Biotechnology Resource Center, Cornell University. The Percheron (PER), Miniature and Arabian horse (AMH) had a library read length of 100 bp and an average insert size of 188 bp, 181 bp and 181 bp respectively. On the other hand, the Brazilian Mangalarga Marchador (MM), a Native Mongolian Chakouyi (CH) and a Tennessee walking horse (TWH) had a library read length of 140 bp and an average insert size of 248 bp, 168 bp and 207 bp respectively.

Read Filtering and Alignment

The raw reads were first inspected using the quality control check program FastQC v10.1 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Then, the reads were quality filtered using Trimmomatic (Bolger *et al.* 2014) which also removed the adapter sequences from the reads. The quality filtering utilized a sliding window of 4 bp and required a minimum mean Phred quality score of 20 within each window. Windows with an average quality less than 20 were sequentially removed from a read. Subsequently, reads with less than 60 bp of sequence remaining were removed from analysis along with their corresponding pairs. The genomes were then aligned to EquCab2 using BWA (Li & Durbin 2009) in the *bwa aln* procedure and the aligned .sai files were combined into Sequence Alignment (SAM) files using *bwa sampe* procedure designated for paired end sequences. The SAM files were sorted and converted to Binary Alignment (BAM) files using PICARD toolkit v1.89 (http://sourceforge.net/projects/picard/) using *SortSam.jar*, then, the duplicate reads in the BAM file were removed using *MarkDuplicates.jar* in the same toolkit. The Genome Analysis Toolkit (GATK) v2.6-5 (DePristo *et al.* 2011), procedures *RealignerTargetCreator* and *IndelRealigner,* were used to perform local realignment of the BAM file reads around the INDELs in order to correct misalignments due to the presence of INDELs.

Base Quality Score Recalibration and Calling SNPs and small INDELs

SNPs and small INDELs (<50bp) were detected using the GATK *HaplotypeCaller* procedure (Van der Auwera *et al.* 2013). The GATK HaplotypeCaller was designed to be very permissive so that it did not miss rare variants. In order to recalibrate base quality scores we used the *BaseRecalibrator* procedure in GATK. Since we do not currently have a gold standard set of variants for the horse (required by the procedure), we undertook an iterative approach (described in the GATK best practices (version 2.4-3). The approach simultaneously recalibrated base quality scores and eventually resulted in the final set of variants. First, the GATK *HaplotypeCaller* procedure (Van der Auwera *et al.* 2013) obtains an initial set of variants subsequently used to recalibrate base quality scores and generate recalibrated BAM files for each genome. The recalibrated files were then used to call variants in the next iteration. Subsequently, variants called in each iteration were used as a bootstrap set in place of gold standard variants to recalibrate the base quality scores in the following iterations. The procedure was iterated until the number of variants and the base quality score recalibrations stabilized, which in our pipeline occurred following the fifth iteration. After that, we used the GATK *VariantRecalibrator* procedure to recalibrate the variants using polymorphisms obtained from the horse genome assembly project as training set (www.ncbi.nlm.nih.gov/projects/SNP/). The VariantRecalibrator algorithm is designed to assign probabilities and quality scores used to filter out those false positives using a statistical machine learning approach. The algorithm learns the best quality score filters based on the data itself and allows the user to trade off sensitivity and specificity. It builds a Gaussian mixture model which uses variants from the input set that overlap variants in the training set. Once the model is trained, variants in the input set that have desired properties as determined by the Gaussian mixture model were filtered using the *ApplyRecalibration* procedure. After careful examination of the tranches plot (resulting from *ApplyRecalibration*) a tranches filter level of 99 was used (**Figure 2.1**). This tranches level was chosen because it resulted in the highest number of true positive SNPs while minimizing false positives.

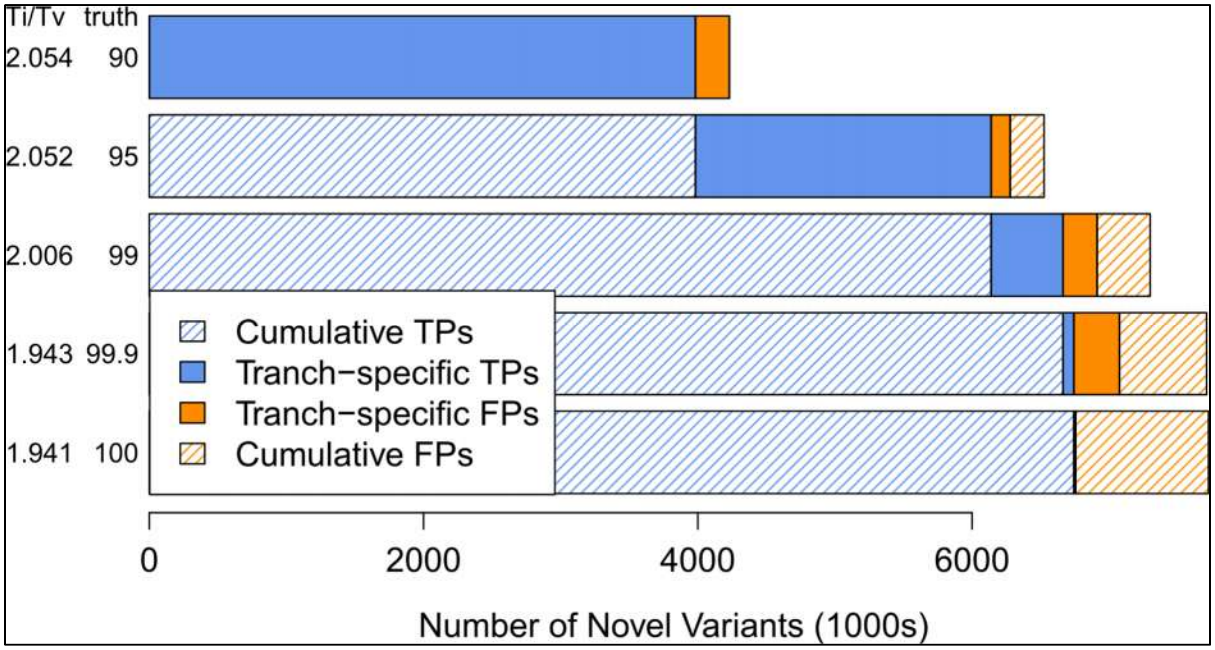


Figure 2.1: Tranches plot generated by GATK VariantRecallibration procedure**.** The plot shows the trade off in (gain in cumulative false positives (FP)) resulting from choosing a certain level of cumulative true positive (TPs) variants. Tranche specific true positives and false positives are shown in blue and orange respectively.

Identifying Structural Variations and Copy Number Variations

The structural variations (SVs) and large INDELs were identified using SVDetect (Zeitouni *et al.* 2010). The program uses anomalously mapped read pairs to localize rearrangements within the genome and classify them into their various types. After filtering out correctly mapped pairs, we used a sliding window of size $2\mu + 2\sqrt{2\sigma}$ to partition the genome, where μ is the estimated insert size and σ is the standard deviation. The length of steps in which the sliding window moved across the genome were set to half of the window size. Control-Freec (Boeva *et al.* 2012) was used to detect copy number variations (CNVs). The program uses GC-content and mapability profiles to normalize read count and therefore gives a better estimate of copy number profiles in high GC or low coverage regions (Boeva *et al.* 2012). A breakpoint threshold of 0.6 and a coefficient of variation of 0.05 were used in the analysis.

Variant Annotation

We used SNPEff v4.0 (Cingolani *et al.* 2012) to annotate the SNPs and short INDELs using the latest available ENSEMBL gene annotation database (EquCab2.76). The output of SNPEff is a full list of effects per variant. SNPs and Indels located within 5,000 bases (5 kb) upstream or downstream genes as well as those within exons, introns, splice sites, and 5' and 3' untranslated regions (UTRs) were also annotated. Since SNPEff output can be integrated into GATK VCF file, we have produced an annotated version of the GATK VCF file which can be loaded and viewed easily in genome browsers. The CNVs and SV breakpoints overlapping ENSEMBL genes were detected using Bedtools (v2.23.0). Ensembl gene IDs were then converted to gene names using Biomart.

We used the Nucleotide Diversity (π) to identify candidate regions targeted by selection using an empirically based outlier approach described in (Kolaczkowski *et al.* 2011). For each of the genomes, the nucleotide diversity (π) was calculated for the SNPs in 1 MB non-overlapping windows using VCFtools v1.10 (Danecek *et al.* 2011). Regions in the lower 1% tail of the π distribution were considered under positive or balancing selection. Genes in these regions were annotated for biological process, using Panther v10.0 (Mi *et al.* 2013). Circos plots (Krzywinski *et al.* 2009) summarizing the distribution of the genomic variations were then created for each of the genomes and a summary circos plot was created to highlight variants in common between the six genomes. To enhance visualization, we removed the small intrachromosomal elements (endpoints size <10 bp) and interchromosomal elements (endpoints size <500 bp) due to their abundance in the output which makes it difficult to visualize in the circos plot.

RT q-PCR analysis of the Latherin CNV

We used Quantitative PCR to quantify the copy number variation within each exon in the horses included in this experiment, the EquCab 2.0 reference genome horse and a control horse. Primers were targeted within exons overlapping the copy number variation and were designed in Primer3 (Untergasser *et al.* 2012) (**Table 2.1**). Genomic DNA (25 ng) was amplified in 10 uL reactions using the Quanta Biosciences PerfeCta SYBR Green (FastMix) as per the manufacturers recommended conditions (Gaithersburg, MD, USA). *ASIP* exon 2 was amplified as reference single-copy gene. Thermocycling and detection were performed using PCR on the Illumina Eco Real-Time PCR System using parameters recommended for the Quanta Mix (58°C annealing). Copy numbers were calculated relative to the reference genome horse. We substituted the Percheron and American Miniature horses by horses from the same breed, as DNA samples from the original two horses was unavailable.

Table 2.1: Real-time quantitative PCR primers.

Gene	Forward primer	Reverse primer	Amplicon size (BP)
LATH	AGGACTCCTTGACGGGAACT	AGGGCCAACCAAGATGTTC	112
BPIFA1	GGAGAAGCACTCACCAGCTC	CTCCAGAGTTCCCGTTTCCT	207
BPIFB4	TGTTGGTGGTGTTCCCTACA	TAGTCGCCATTTCGAAGGTC	198
BPIFA2	CGTTTTTGTCAGGTGTCTTCC	CCCAAAGAACCATCCACAGT	157

RESULTS AND DISCUSSION

Whole genome sequencing and alignment

Sequencing was completed using the Illumina HiSeq2500 (Illumina, San Diego, CA) with manufacturer recommended reagents and procedures by the Biotechnology Resource Center at Cornell University. The number of the paired-end reads before and after filtering and their corresponding depth of coverage values are given in **Table 2.2**. The raw number of reads resulting from sequencing the six horses ranged between 324,123,384 reads on the American miniature to 142,502,233 reads on the Native Mongolian horse. After filtering the reads, between 83,123,251 reads (Mangalarga Marchadore) and 187,223,705 reads (Percheron) were retained. This corresponded to an average depth of coverage of 6.16 x to 13.87 x on the filtered reads. After mapping, the average depth of coverage ranged from 10.03 to 16.7845x (**Table 2.2**). The percentage of reads where both pairs successfully mapped were between 91.57% and 97.20%, which indicates a fairly successful mapping procedure comparable to previous studies (Doan *et al.* 2012). A diagram summarizing the process of reads filtering, mapping, variant identification and the tools used in each step is shown in **Figure 2.2**.

Table 2.2: Yield, filtering and mapping summary of the next generation sequencing data of six horses from different breeds.

	Arabian	**Percheron**	**American Miniature**	**Tennessee Walking**	**Mangalarga Marchador**	**Native Mongolian Chakouyi**
Number of paired end reads before trimming	241,480,555	296,460,133	324,123,384	198,749,393	169,680,137	142,502,233
Read lengths	100/100	100/100	100/100	150/150	150/150	150/150
Estimated average depth of coverage before trimming[1]	17.8x	21.96x	24x	14.72x	12.57x	10.56x
Number of paired end reads after trimming	165,277,009	187,223,705	138,772,441	161,659,278	83,123,251	121,744,242
Estimated average depth of coverage after trimming[1]	12.24x	13.87x	10.28x	11.97x	6.16x	9.02x
Total number of aligned reads	330,554,018	374,447,410	277,544,882	323,318,556	269,464,788	243,488,484
Percentage of mapped reads	98%	93%	93%	97%	95%	96%
Percentage of reads where both pairs mapped	97%	92%	92%	96%	94%	94%

1. Estimated using the formula C= L*N/G

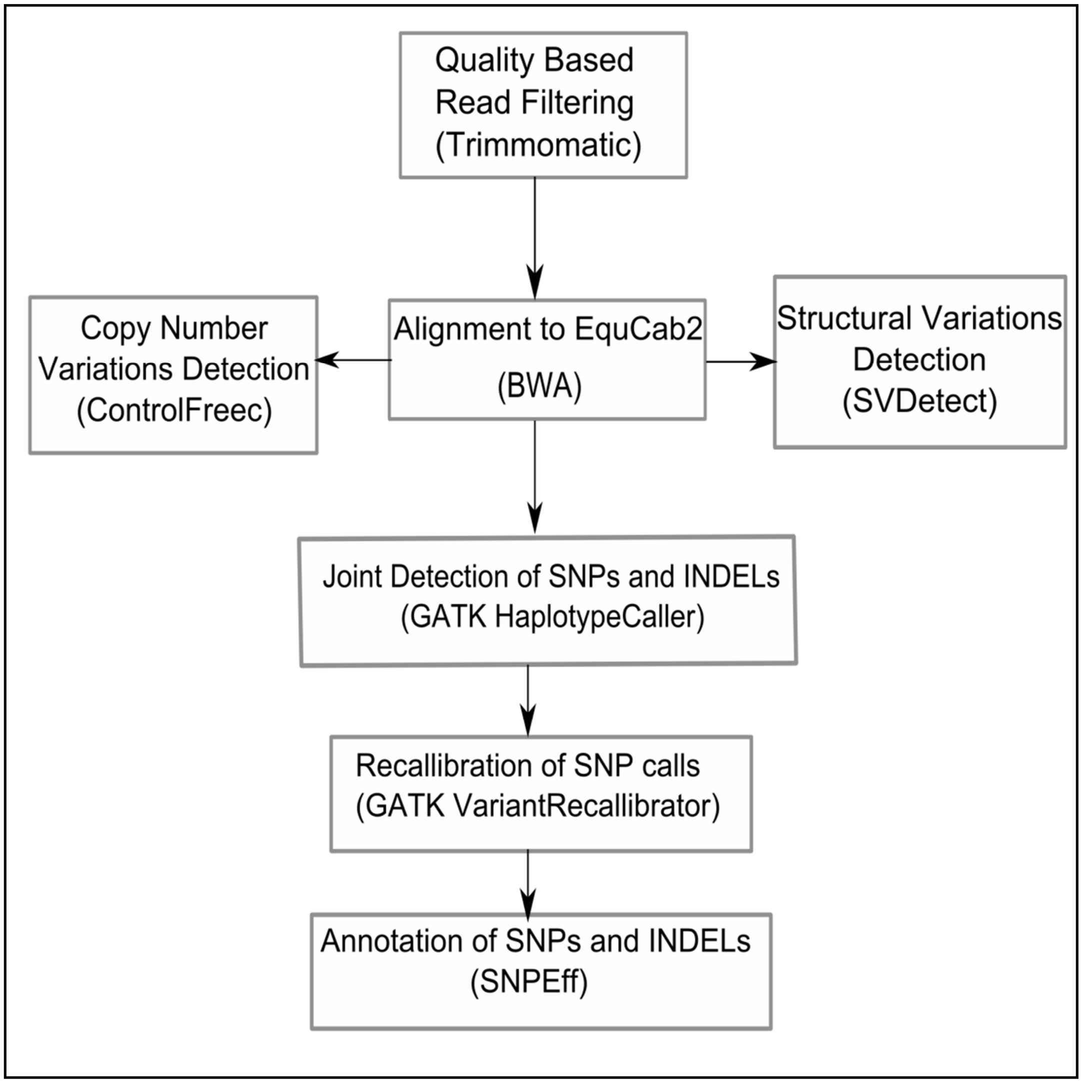


Figure 2.2: An overview of the pipeline used in the reads processing and variant detection. Description of the step and the name of the program/software given in parenthesis.

Identification of Variants

SNPs and INDELs

In total, 8,562,696 SNPs were detected using the GATK *HaplotypeCaller*. These were processed using the GATK *VariantRecalibrator* procedure, producing a final set of 8,128,658 SNPs. The number of SNPs is about 0.3 % of the size of the genome (about 1 every 300 base pairs) which is very similar to the percentage of SNPs estimated in the human genome (Gibbs *et al.* 2003). Amongst those, 11,537 SNPs (0.14 %) were multi-allelic. The mean transition to transversion ratio in these horses is 1.998 (range 1.991 to 2.008) (**Table 2.3**) which is very similar to other mammalian species (Abecasis *et al.* 2012). The allelic frequency spectrum (**Figure 2.3**) showed an expected decline in the frequency of SNPs as the observed number of the alternative allele increased, as observed in other studies (Manske *et al.* 2012),(Gravel *et al.* 2011). The mean, median and standard deviation of Phred-scaled quality scores for the SNPs were 785.78, 543 and 732.85, respectively, which signifies a very high call accuracy. Relative to the chromosome size, the highest proportion of SNPs was found in chromosome 12 (0.5 %) followed by chromosome 20 (0.4%) (**Figure 2.5**).

Genotype counts of homozygous reference, heterozygous, homozygous alternative, as well as the number of missing SNPs for each of the horses is shown in **Table 2.3**. A close examination of the table reveals that the numbers in each category were generally similar in all horses. The highest numbers of SNPs calls were homozygous reference calls, comprising 43 to 47 % of the genotypes in each horse (**Table 2.3**). An interesting observation is that the highest proportion of homozygous reference genotypes was found in the Arabian horse. This may be explained by the fact that, among the breeds included, the Arabian horse has the closest historical relationship to the reference genome derived from a mare of the Thoroughbred breed. In fact, the Thoroughbred horse population originated by mating three prominent Arabian stallions to native mares in England during the 17th century (Bower *et al.* 2012).

Table 2.3: Genotype categories of SNPs and INDELs and counts of CNVs and SVs in the six horses.

	Arabian	**Percheron**	**American Miniature**	**Tennessee Walking**	**Mangalarga Marchador**	**Native Mongolian Chakouyi**
SNPs						
Homozygous Reference	3861988	3549746	3814288	3530966	3731291	3577545
Heterozygous	2328125	2491424	2266059	2658622	2387676	2513707
Homozygous Alternative	1907689	2054426	1998897	1922879	1954411	1989304
Missing	30856	33062	49414	16191	55280	48102
Transitions	4090739	4406054	4179785	4335010	4194652	4321840
Transversions	2052764	2194222	2084068	2169370	2101846	2170475
INDELs						
Homozygous Reference	272640	254728	276892	255475	277540	244701
Heterozygous	193566	198211	182198	208226	189440	210612
Homozygous Alternative	356999	370374	357710	359412	337514	360065
Missing	7165	7057	13570	7257	25876	14992
CNVs	999	1007	923	976	934	706
Gains	854	863	776	814	794	613
Losses	145	145	147	162	141	96
Structural Variations	3166	4072	10707	4385	8296	1394
Interchromosomal	178	201	116	708	1495	198
Intrachromosomal	2988	3871	10591	3677	6801	1196

A comparison of these SNPs with the horse SNP database in dbSNP (www.ncbi.nlm.nih.gov/projects/SNP/) and Ensembl (ftp://ftp.ensembl.org/pub/release80/variation/vcf/equus caballus/Equus caballus.vcf.gz) showed that 5,221,242 novel and 2,907,416 known variants (**Figure 2.4**). Two of the sequenced horses were genotyped previously using the Illumina EquineSNP50 array (Illumina Inc.) enabling a test of the genotype concordance between the two methods. The concordance of the genotypes detected using the Equine SNP50 genotyping array and those detected by NGS was 96% for the American Miniature and 98% for the Percheron horse, illustrating that the SNPs detection is comparable to array-based methods and is reliable for the purposes of this study.

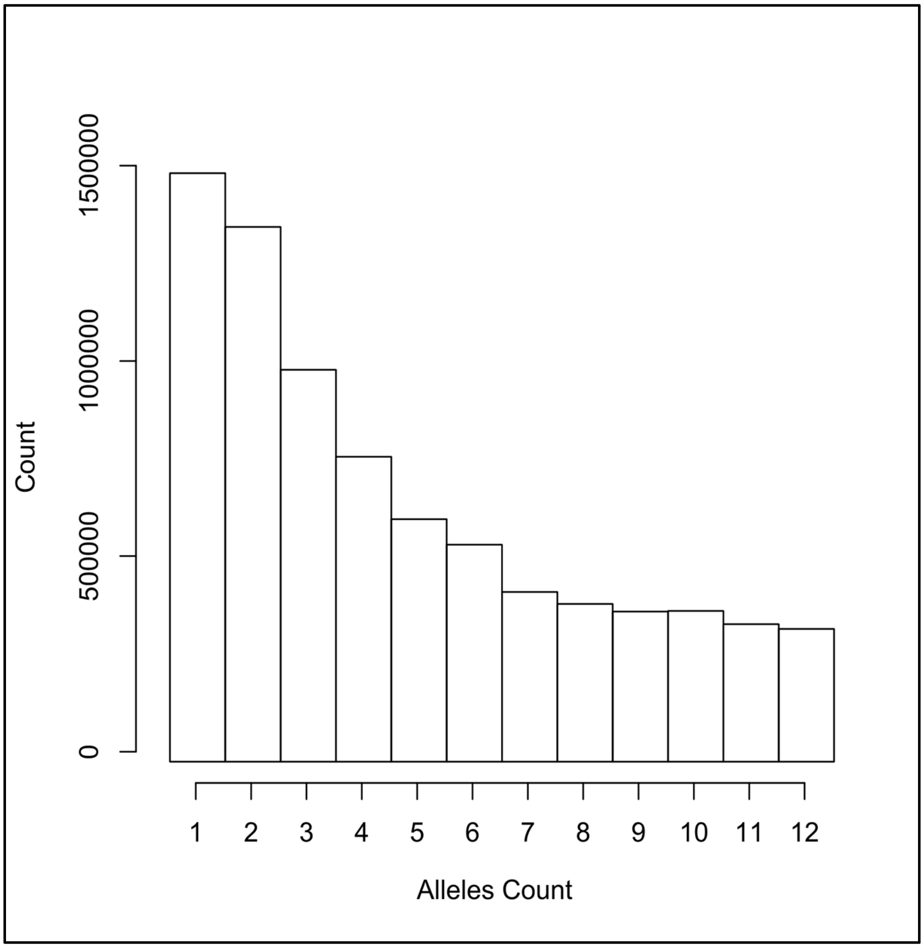


Figure 2.3: The allele frequencies of SNPs from whole-genome sequence data of the six horses showing a lower frequency as observations of the alternate allele increased.

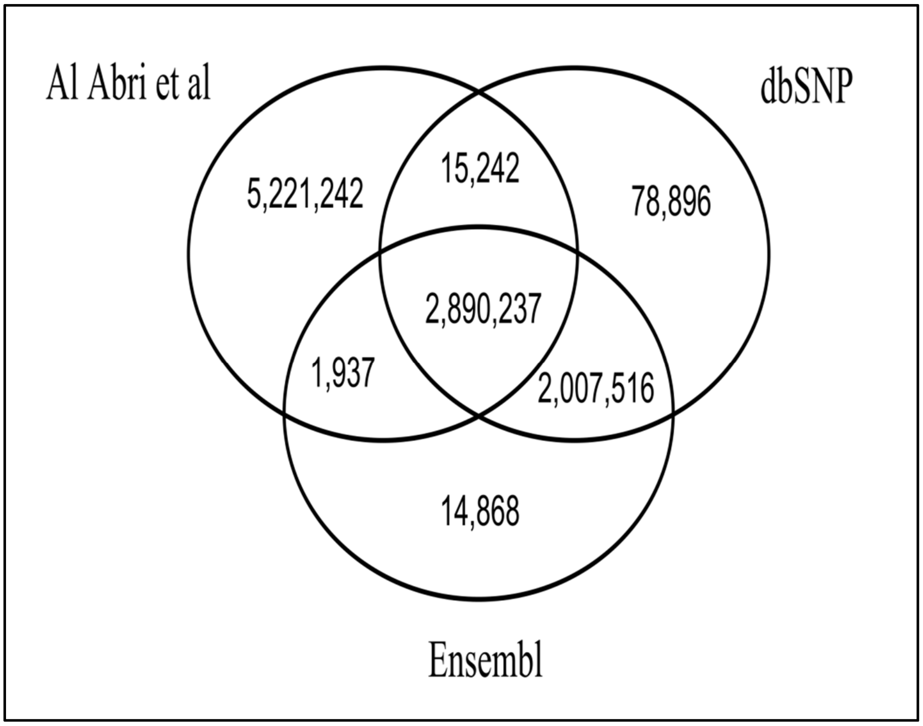


Figure 2.4: Comparison of SNP data detected in the present study with SNPs currently deposited Ensembl and dbSNP databases. The present study was the highest in terms of the number of private SNPs.

It is well established that INDELs are the second most common form of genomic variations, altering a similar total proportion of base pairs as SNPs (Mullaney *et al.* 2010). We detected 830,370 small INDEL loci jointly with the SNPs in the GATK *HaplotypeCaller* procedure. Within this set, 10,811 INDELs were multi-allelic. The mean, median and standard deviation of Phred-scaled quality scores for the INDELs were 1,025, 785 and 1,076 respectively which signifies a higher accuracy, but more dispersion in accuracy, to their SNPs counterparts. The INDELs size ranged between 0 and 219, with mean of 1.164 bp and the majority of INDELs were < 10 bp. The INDELs were split almost equally between insertions and deletions (48 % and 52 % respectively), as is observed in the pattern of INDELs in humans (Mills *et al.* 2006), (Bhangale *et al.* 2005). Unlike SNPs, the most frequent small INDELs calls were the homozygous alternative calls which ranged between 40 and 44 % of the total INDELs calls in different horses. INDELs are more rare events than SNPs and are thus more likely to be unique to a breed of horses than to be shared between breeds (Ajawatanawong & Baldauf 2013). In fact, the resolution of the Eukaryotic phylogenetic tree can be improved by incorporating INDELs (Bapteste & Philippe 2002). It is noteworthy to indicate that the incidence of homozygous alternative (non-reference homozygous) SNPs and INDELs genotypes was highest in the Percheron horse. This could be a result of a larger evolutionary distance between the reference genome and the Percheron relative to the other breeds. The SNPs and INDELs missingness was the highest in the Mangalarga Marchadore (the sample with the lowest coverage) and was inversely correlated with the aligned read depth (**Tables 2.2 and 2.3**).

CNVs and SVs

CNVs and SVs are often complex and may contain DNA sequence belonging to different sites in the genome. However, genome-wide datasets produced by NGS technologies are revealing a wealth of knowledge about their frequency and structure. The number of CNVs and SVs in various horses is given in **Table 2.3**. Of the identified CNVs, the number of gains was consistently higher than the number of losses for all horses. Since many of the gains are shared between horses, we hypothesize that the excess of gains is an artifacts of the computational assembly of EquCab2.0, compressing regions of repetitive sequences and highly homologous gene families. Numerous regions (or genes) in the genome could be actual duplication events, yet a failure to assign these sequences to their correct locus, often annotating them as ChrUn by default, has rendered them difficult to study.

Additionally, we also observe a consistent excess of intrachromosomal SVs compared to the interchromosomal SVs (**Table 2.3**). Bias towards intrachromosomal SVs is not uncommon in this type of analysis and is often due to intrachromosomal joining bias resulting from the relative closer proximity of genomic regions and has been observed studies of the mouse (Klein *et al.* 2011), humans (Lieberman-Aiden *et al.* 2009) and chicken (Bourque *et al.* 2005). It is proposed that a biological mechanism preferring proximal intrachromosomal rearrangement reduces large-scale genomic alterations, and therefore maintains genomic stability (Klein *et al.* 2011).

Compared to other horses, the American miniature horse possessed the highest number of the Intrachromosomal SVs (n= 10591) and the Mangalarga Marchador the largest number of Interchromosomal translocations (n=1495). These results are more than double the average of the corresponding values in the other horses and may be an artifact of imperfect library preparation or fragment size selection prior to sequencing. Indeed, filtering of such artifacts is a significant challenge for reliable discovery and genotyping of SVs by sequence based methods. We formatted our SVs into two separate tracks for inter- and intra- chromosomal translocations with different colored assigned to different SV types. Interchromosomal translocations were formatted into a click button format such that clicking on the feature link the user to the chromosomal address of the other end of the feature. For the intrachromosmal translocations, the putative breakpoints of the feature are displayed in a GFF style joined together. The CNVs were formatted into a bed format with different colors for gains, losses and normal copy numbers. These tracks are available for download at http://www.animalgenome.org/repository/horse and can be loaded directly into UCSC genome browser. The resulting CNVs and SVs were annotated using the ENSEMBL genes they overlap with and the results can also be downloaded at http://www.animalgenome.org/repository/horse.

Annotation of Detected Variants

The majority of SNPs were intergenic, followed by intronic, comprising 60 % and 27 % of SNPs, respectively (**Table2.4**) (Zhao *et al.* 2003). The small proportion of exonic SNPs likely results from strong negative selective pressure exerted on coding regions due to functional implications of these alterations (Bhangale *et al.* 2005). Likewise, lower diversity was observed for SNPs around 3' UTR, 5' UTR and coding regions compared to other regions which was also reported in other studies (Zhao *et al.* 2003) (**Table2.4**). A lower allelic diversity within the 5' UTRs and around coding regions was also observed in the INDEL category of polymorphisms, a phenomenon also found in studies of the human genome (Bhangale *et al.* 2005).

Table 2.4: Annotation of SNPs and INDELs by type in the six horses genomes.

	SNPs		INDELs	
Effects according to region				
Exon	7383	0.08%	618	0.06%
Intergenic	5760944	60.29%	583364	57.94%
Intron	2601880	27.23%	289589	28.76%
3’-UTR	4665	0.05%	707	0.07%
5’-UTR	1657	0.02%	418	0.04%
Downstream	286342	3.00%	49622	4.93%
Start lost	28	0.00%	1	0.00%
Stop gained	242	0.00%	5	0.00%
Upstream	413882	4.33%	48894	4.86%
Unclassified	286342	3.00%	26758	2.66%
Effects according to functional impact (SNPs only)				
Non synonymous coding	26322	0.28%		
Non synonymous start	4	0.00%		
Splice site acceptor	187	0.00%		
Splice site donor	269	0.00%		
Splice site region	6188	0.07%		
Start gained	255	0.00%		
Stop lost	17	0.00%		
Synonymous coding	33593	0.35%		
Synonymous stop	15	0.00%		
Effects according to functional impact (INDELs only)				
Codon change plus codon deletion	55	0.01%		
Codon change plus codon insertion	37	0.00%		
Codon deletion	83	0.01%		
Codon insertion	80	0.01%		
Frame shift	2863	0.28%		
Frame shift+start lost	5	0.00%		
Frame shift+stop gained	11	0.00%		
Frame shift+stop lost	3	0.00%		
Intragenic	3	0.00%		
Splice site acceptor	669	0.07%		
Splice site donor	645	0.06%		
Splice site region	2396	0.24%		

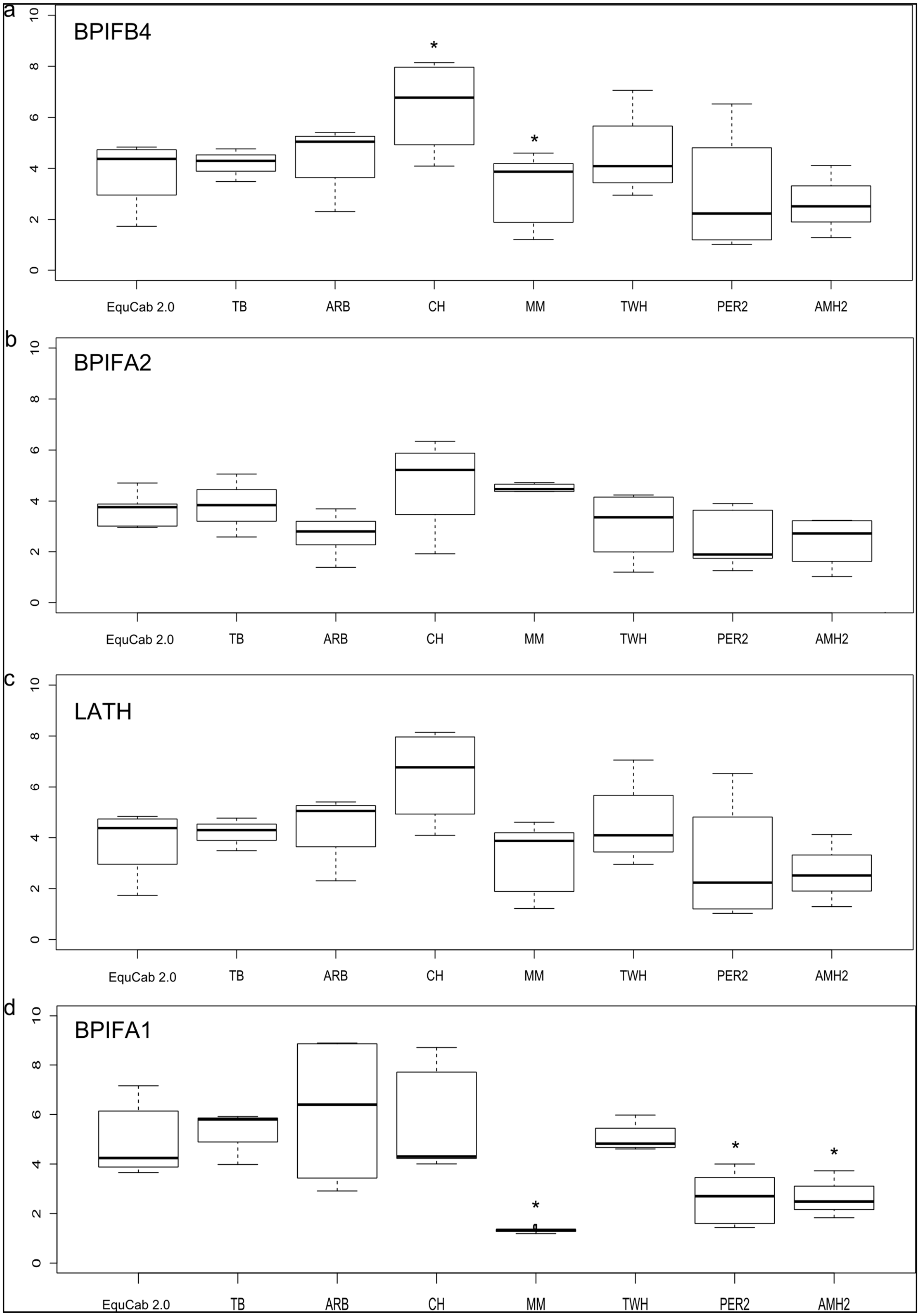
In terms of predicted functional impact, the majority of SNPs found in coding sequences (33593, 0.35%) were likely synonymous. Yet, 26322 (0.28 %) SNPs are predicted to result in a non-synonymous amino acid change. This proximity in percentage of Synonymous and non-synonymous SNPs was previously reported in the quarter horse (Doan *et al.* 2012). On the other hand, 2863 (0.28%) INDELs caused frame-shifts, which is number very similar to that obtained in a previous study in the Quarter horse genome (Jun *et al.* 2014),(Doan *et al.* 2012).

Copy number variation and structural variations are given relatively less attention than SNPs in studies of diversity. Nevertheless, they are ubiquitous in the horse genome and influence a number of phenotypes (Gizaw *et al.* 2013; Wang *et al.* 2014). We found that chromosomes 12 and 20 had the highest density of CNVs. Functional annotation of these regions revealed genes involved in olfactory reception and immunity which are also enriched in genes overlapping human CNVs (Nguyen *et al.* 2006). This observation was previously reported in a CNV an analysis of six horse breeds (Wang *et al.* 2014). Additionally, our CNV annotation showed a copy number gain in a gene cluster that includes latherin gene in all the horses in this study. This copy number gain was previously reported in the quarter horse using NGS data (Doan *et al.* 2012), although using array CGH a copy number loss was observed in the same region (Wang *et al.* 2014). LATH (also known as BPIFA4) is a member of the palate lung and nasal epithelium clone (PLUNC) family of proteins that is common in the oral cavity and saliva of mammals (Bingle *et al.* 2011; Vance *et al.* 2013). In horses and other equids this gene produces a surfactant protein that is expressed in the saliva and sweat (McDonald *et al.* 2009). Equine latherin protein is postulated to play a role in mastication of fibrous food and evaporative cooling in horses (Vance *et al.* 2013). Therefore, it is reasonable to postulate that the gain in LATH copies observed in this study results from an evolutionary pressure for improved evaporative dissipation of heat, yielding athleticism and endurance in hot environments.

Our RT-qPCR analysis of the CNV region in a number of horses (**Figure 2.5)** revealed evidence of between 2 and 6 copies of *LATH* relative to a single copy control gene. Our analysis also suggested polymorphism in the number of copies of nearby genes *BPIFB4*, *BPIFA2* and *BPIFA1*. Accumulation of copies of these genes could be an adaptation to improve evaporative cooling. Validation of this CNV polymorphism is challenging due to a poor quality of assembly, a complex structure within that part of the genome, and the technical limitations of qPCR. Thus, precise determination of polymorphisms in *LATH* will require more precise techniques like digital qPCR (Baker 2012).

Our annotation of the SVs showed duplication events within the *ZFAT* gene (ECA 9) unique to the American Miniature horse. *ZFAT* gene was previously shown to be associated with withers height and overall skeletal size in horses (Makvandi-Nejad *et al.* 2012; Signer-Hasler *et al.* 2012). We also detected an inverted duplication unique to the Percheron horse in *HMGA1*. *HMGA1* appears to play a role in overall body size. *HMGA1* showed a reduced body weight and size compared to wild type mice (Federico *et al.* 2014). These small SVs may impact regulatory motifs in these genes, leading to the size phenotypes observed in these breeds, though additional work is required to investigate this.

Figure 2.5: RT-qPCR results of the *LATH* CNV region. Results for different primers are shown relative to their position in the genome. Evidence of a copy number variation is seen in *BPIFB4* (**a**) and *BPIFA1* (**d**) genes that flank *LATH* (**c**).


a
BPIFB4
b
BPIFA2
c
LATH
d
BPIFA1
0
2
4
6
8
10
EquCab 2.0
TB
ARB
CH
MM
TWH
PER2
AMH2
*

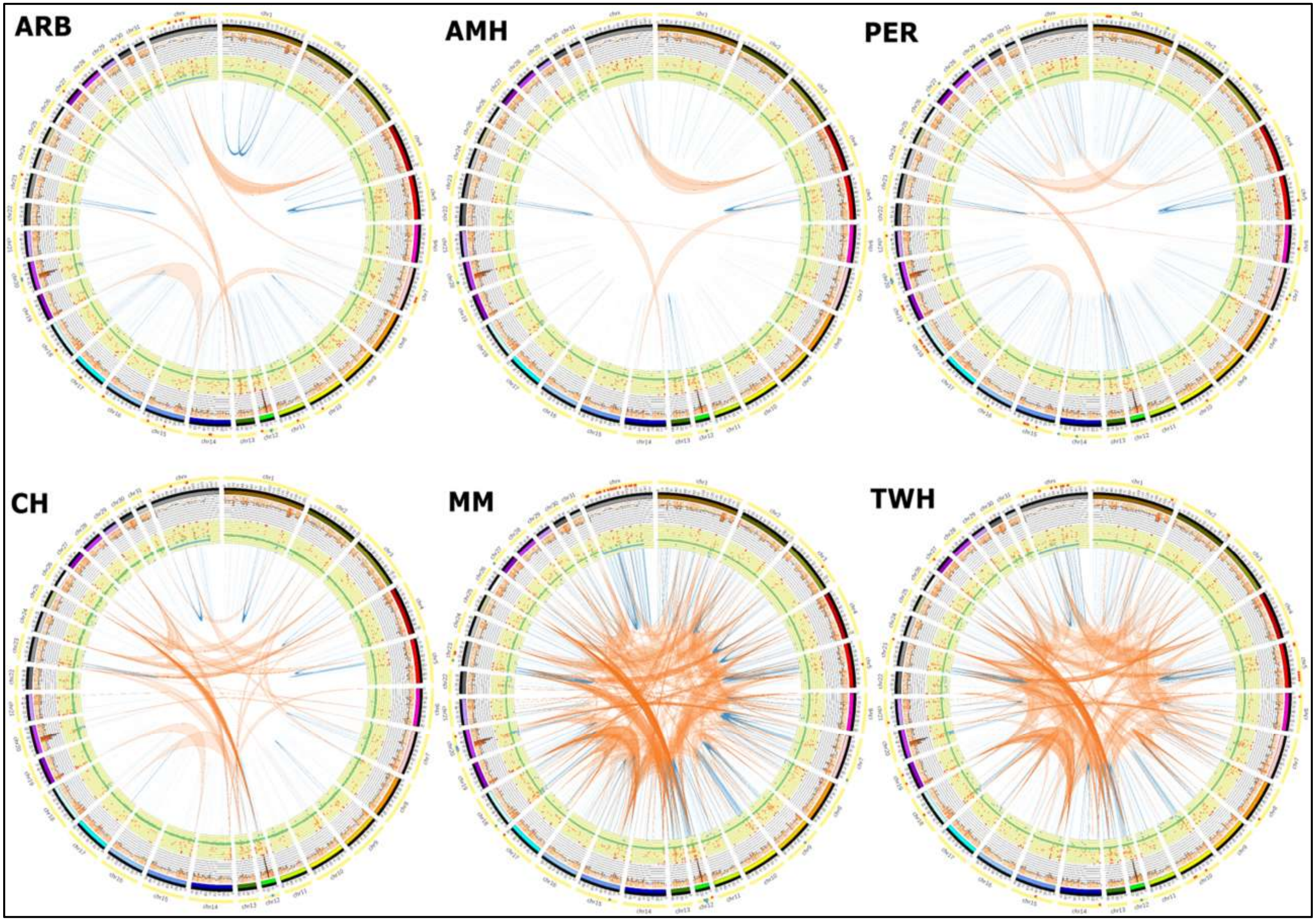
Genome-wide diversity (π)

Nucleotide diversity (π) (Nei & Li 1979) is defined as the average number of nucleotide differences per site between two randomly chosen sequences in a population. Assessment of nucleotide diversity provides a valuable insight into the divergence of populations, inferring the demographic history of the species, as well as the historical size of the population (Yu *et al.* 2004). Areas of lower than expected nucleotide diversity may signify signatures of past selection events (Quach *et al.* 2009). In order to find such regions we calculated the nucleotide diversity (π) for the resulting SNPs belonging to each horse using 1 megabase (MB) non-overlapping windows.

The average nucleotide diversity across all six horses was 0.00097 for all SNP polymorphisms, and ranged from a minimum of 0.00090 for the American Miniature Horse and 0.0011 for the Tennessee Walking Horse. This could reflect a higher inbreeding in the American Miniature horse compared to the Tennessee Walking Horse sequenced in this study. Average diversity in the autosomal chromosomes for all horses was 0.0010, which is four times as high as the mean diversity observed in the X chromosome (0.00026). Since the X chromosome has three-quarters the effective population size (N_e) of that of the autosomes, lower nucleotide diversity for the X chromosome is to be expected. However, a lower diversity level could also be due to a lower mutation rate (μ). Besides that, the fact that the reference genome was based on a female horse has largely impacted the nucleotide diversity levels in the male horses used in this study (**Figure 2.6**). It is expected to observe more differences (homozygous alternate SNPs) between the reference genome X chromosome and two copies of the X chromosomes in female horses compared to males horses with only one copy.

Notably, the SNP dense region on ECA20 and ECA12 were amongst the highest 1% regions in nucleotide diversity (π) (**Figure 2.6**). PANTHER statistical over-representation analysis of genes in these regions revealed that they are enriched for immune response and immunological response and antigen processing (ECA20) and metabolic and sensory perception in (ECA12). On the other hand, among the lowest 1% of the empirical distribution of π values, the most represented GO terms categories in all six horses were metabolic process (42.32 %) followed by cellular process (29 %) and biological regulation (18.45 %). Remarkably, statistical over-representation analysis of those regions in respective horses showed enrichment for skeletal and digestive system development in the Percheron horse. Regions with lowest 1% π values in the Percheron included *HOXD12*, *MYO3B*, and *HOXD1* on ECA 18 and *ANKRD1* (ECA 1). *HOXD12* and *HOXD1* belong to the *HOX* family of transcription factor genes are known for their role in skeletal and limb development (Knezevic *et al.* 1997; Pitera *et al.* 2001; Di-Poï *et al.* 2009). *ANKRD1* is a transcriptional factor to the muscle ankyrin repeat proteins (MARP) family (Duboscq-Bidot *et al.* 2009) and linked to size by GWAS in chapter two of this work. MARP proteins are expressed in developing skeletal muscles and are important for muscle development (Baumeister *et al.* 1997).On the other hand Myo3B belongs to the class III myosin genes and is expressed primarily in the retina but is also expressed in the kidney, and testis (Dose & Burnside 2002; Dosé *et al.* 2003).

Figure 2.6: Circos plot summarizing the genetic variants detected in each horse. From the inside out, each plot shows two endpoints of the inter- (orange) and intra- (blue) chromosomal translocations. Intrachromosomal translocations > 5MB are in dark blue. The yellow ring shows the copy number variations (green =normal, blue = loss, red=gain). The histogram (in orange) shows the density of SNPs detected using 1MB windows. The outermost track in yellow marks the lower 1% (red) and upper 1% (blue) values of the average nucleotide diversity calculated using 1 MB windows. Lower nucleotide diversity levels in the X chromosomes can be seen in male vs female horses. The dense clustering of SNPs amidst chromosomes 12 and 20 was expected given that both chromosomes contain structurally complex regions important for immunity. To enhance visualization, intrachromosomal translocations with end points <10bp and interchromosomal translocations with end points < 500 bp are not displayed here.


ARB
AMH
PER
CH
MM
TWH

.

We compared our findings with two previous studies that investigated signatures of selection in the horse. The first study (Petersen *et al.* 2013) used an Illumina SNP50 Beadchip to scan the genome of multiple breeds using an F_{ST}-based statistic, while the second study compared NGS data on ancient Przewalski's horses to modern domesticated horses to pinpoint selection signatures in modern breeds (Schubert *et al.* 2014). We found no overlap between genes under selection found in this study and those reported in (Petersen *et al.* 2013) possibly due to the technical difference and genome coverage between NGS used in this study and the Illumina SNP50 Beadchip. However, three genes under selection reported in (Schubert *et al.* 2014) were also found in this study, namely *NINJ1* and *SEC63* in the Tennessee walking horse and *COMMD1* in the Arabian horse. *NINJ1* codes for ninjurin a protein that is highly expressed in human brain endothelial (Ifergan *et al.* 2011) and becomes up-regulated after nerve injuries in Schwann cells and in dorsal root ganglion neurons (Araki & Milbrandt 1996). *SEC63* encodes a membrane protein of the Endoplasmic Reticulum which is highly conserved in humans and is part of protein translocation apparatus of the endoplasmic reticulum (Davila *et al.* 2004). Certain mutations in SEC63 cause autosomal dominant polycystic liver disease in humans (Davila *et al.* 2004). On the other hand, *COMMD1* is involved in copper storage in dogs and copper-storage disorder in Bedlington terriers is a known autosomal recessive disorder that causes rapid accumulation of copper in the liver of affected dogs (Fedoseienko *et al.* 2015).

CONCLUSION

We present a next generation sequencing and variants detection analysis of six horses belonging to six different breeds. Functional annotation of the detected variants was indicative of selection pressure for specific phenotypic characteristics in the breeds of horses included in this study. We detected a copy number gain in the Latherin gene common to all horses that could be the result of an evolutionary selection for athleticism and heat tolerance. Our results also revealed putatively functional variants unique to each horse including *HOXD12* and *HOXD1* and *ANKRD1* and *HMGA1* in the Percheron and *ZFAT* gene in the American Miniature horse. Our results also showed a copy number gain of genes involved in immunity and olfactory reception in ECA 20 ECA 12 respectively.

REFERENCES

Comprehensive identification and characterization of diallelic insertion-deletion polymorphisms in 330 human candidate genes.

Abecasis G.R., Auton A., Brooks L.D., DePristo M.A., Durbin R.M., Handsaker R.E., Kang H.M., Marth G.T. & McVean G.A. (2012) An integrated map of genetic variation from 1,092 human genomes. *Nature* **491**, 56-65.

Ajawatanawong P. & Baldauf S.L. (2013) Evolution of protein indels in plants, animals and fungi. *BMC evolutionary biology* **13**, 140-.

Araki T. & Milbrandt J. (1996) Ninjurin, a novel adhesion molecule, is induced by nerve injury and promotes axonal growth. *Neuron* **17**, 353-61.

Baker M. (2012) Digital PCR hits its stride. *Nature methods* **9**, 541-4.

Bapteste E. & Philippe H. (2002) The Potential Value of Indels as Phylogenetic Markers: Position of Trichomonads as a Case Study.

Baumeister A., Arber S. & Caroni P. (1997) Accumulation of Muscle Ankyrin Repeat Protein Transcript Reveals Local Activation of Primary Myotube Endcompartments during Muscle Morphogenesis.

Bhangale T.R., Rieder M.J., Livingston R.J. & Nickerson D.A. (2005) Comprehensive identification and characterization of diallelic insertion-deletion polymorphisms in 330 human candidate genes. *Hum Mol Genet* **14**, 59-69.

Bingle C., Seal R. & Craven C. (2011) Systematic nomenclature for the PLUNC/PSP/BSP30/SMGB proteins as a subfamily of the BPI fold-containing superfamily. *Biochem Soc Trans* **39**, 977-83.

Boeva V., Popova T., Bleakley K., Chiche P., Cappo J., Schleiermacher G., Janoueix-Lerosey I., Delattre O. & Barillot E. (2012) Control-FREEC: a tool for assessing copy number and allelic content using next-generation sequencing data. *Bioinformatics (Oxford, England)* **28**, 423-5.

Bolger A.M., Lohse M. & Usadel B. (2014) Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics (Oxford, England)* **30**, 2114-20.

Bourque G., Zdobnov E.M., Bork P., Pevzner P.A. & Tesler G. (2005) Comparative architectures of mammalian and chicken genomes reveal highly variable rates of genomic rearrangements across different lineages. *Genome Res* **15**, 98-110.

Bower M.A., McGivney B.A., Campana M.G., Gu J., Andersson L.S., Barrett E., Davis C.R., Mikko S., Stock F., Voronkova V., Bradley D.G., Fahey A.G., Lindgren G., MacHugh D.E., Sulimova G. & Hill E.W. (2012) The genetic origin and history of speed in the Thoroughbred racehorse. *Nature communications* **3**, 643.

Cingolani P., Platts A., Wang le L., Coon M., Nguyen T., Wang L., Land S.J., Lu X. & Ruden D.M. (2012) A program for annotating and predicting the effects of single nucleotide polymorphisms, SnpEff: SNPs in the genome of Drosophila melanogaster strain w1118; iso-2; iso-3. In: *Fly (Austin)* (pp. 80-92, United States.

Daetwyler H.D., Capitan A., Pausch H., Stothard P., Binsbergen R.v., Brøndum R.F., Liao X., Djari A., Rodriguez S.C., Grohs C., Esquerré D., Bouchez O., Rossignol M.-N., Klopp C., Rocha D., Fritz S., Eggen A., Bowman P.J., Coote D., Chamberlain A.J., Anderson C., VanTassell C.P., Hulsegge I., Goddard M.E., Guldbrandtsen B., Lund M.S., Veerkamp R.F., Boichard D.A., Fries R. & Hayes B.J. (2014) Whole-genome sequencing of 234 bulls facilitates mapping of monogenic and complex traits in cattle. *Nature Genetics* **46**, 858-65.

Danecek P., Auton A., Abecasis G., Albers C.A., Banks E., DePristo M.A., Handsaker R.E., Lunter G., Marth G.T., Sherry S.T., McVean G. & Durbin R. (2011) The variant call format and VCFtools. *Bioinformatics (Oxford, England)* **27**, 2156-8.

Davila S., Furu L., Gharavi A.G., Tian X., Onoe T., Qian Q., Li A., Cai Y., Kamath P.S., King B.F., Azurmendi P.J., Tahvanainen P., Kääriäinen H., Höckerstedt K., Devuyst O., Pirson Y., Martin R.S., Lifton R.P., Tahvanainen E., Torres V.E. & Somlo S. (2004) Mutations in SEC63 cause autosomal dominant polycystic liver disease. *Nature Genetics* **36**, 575-7.

DePristo M.a., Banks E., Poplin R., Garimella K.V., Maguire J.R., Hartl C., Philippakis A.a., del Angel G., Rivas M.a., Hanna M., McKenna A., Fennell T.J., Kernytsky A.M., Sivachenko A.Y., Cibulskis K., Gabriel S.B., Altshuler D. & Daly M.J. (2011) A framework for variation discovery and genotyping using next-generation DNA sequencing data. *Nature genetics* **43**, 491-8.

Di-Poï N., Montoya-Burgos J.I. & Duboule D. (2009) Atypical relaxation of structural constraints in Hox gene clusters of the green anole lizard. *Genome Res* **19**, 602-10.

Doan R., Cohen N.D., Sawyer J., Ghaffari N., Johnson C.D. & Dindot S.V. (2012) Whole-genome sequencing and genetic variant analysis of a Quarter Horse mare. *BMC Genomics* **13**, 78-.

Dose A.C. & Burnside B. (2002) A class III myosin expressed in the retina is a potential candidate for Bardet-Biedl syndrome. *Genomics* **79**, 621-4.

Dosé A.C., Hillman D.W., Wong C., Sohlberg L., Lin-Jones J. & Burnside B. (2003) Myo3A, One of Two Class III Myosin Genes Expressed in Vertebrate Retina, Is Localized to the Calycal Processes of Rod and Cone Photoreceptors and Is Expressed in the Sacculus.

Duboscq-Bidot L., Charron P., Ruppert V., Fauchier L., Richter A., Tavazzi L., Arbustini E., Wichter T., Maisch B., Komajda M., Isnard R. & Villard E. (2009) Mutations in the ANKRD1 gene encoding CARP are responsible for human dilated cardiomyopathy. In:

Eur Heart J (pp. 2128-36, England.

Federico A., Forzati F., Esposito F., Arra C., Palma G., Barbieri A., Palmieri D., Fedele M., Pierantoni G.M., De Martino I. & Fusco A. (2014) Hmga1/Hmga2 double knock-out mice display a “superpygmy” phenotype. In: *Biol Open* (pp. 372-8.

Fedoseienko A., Netherlands M.G.s.U.o.G.U.M.C.G.G.t., Bartuzi P., Netherlands M.G.s.U.o.G.U.M.C.G.G.t., Sluis B. & Netherlands M.G.s.U.o.G.U.M.C.G.G.t. (2015) Functional understanding of the versatile protein copper metabolism MURR1 domain 1 (COMMD1) in copper homeostasis. *Annals of the New York Academy of Sciences* **1314**, 6-14.

Gibbs R.A., Belmont J.W., Hardenbol P., Willis T.D., Yu F., Yang H., Ch'ang L.-Y., Huang W., Liu B., Shen Y., Tam P.K.-H., Tsui L.-C., Waye M.M.Y., Wong J.T.-F., Zeng C., Zhang Q., Chee M.S., Galver L.M., Kruglyak S., Murray S.S., Oliphant A.R., Montpetit A., Hudson T.J., Chagnon F., Ferretti V., Leboeuf M., Phillips M.S., Verner A., Kwok P.-Y., Duan S., Lind D.L., Miller R.D., Rice J.P., Saccone N.L., Taillon-Miller P., Xiao M., Nakamura Y., Sekine A., Sorimachi K., Tanaka T., Tanaka Y., Tsunoda T., Yoshino E., Bentley D.R., Deloukas P., Hunt S., Powell D., Altshuler D., Gabriel S.B., Zhang H., Matsuda I., Fukushima Y., Macer D.R., Suda E., Rotimi C.N., Adebamowo C.A., Aniagwu T., Marshall P.A., Matthew O., Nkwodimmah C., Royal C.D.M., Leppert M.F., Dixon M., Stein L.D., Cunningham F., Kanani A., Thorisson G.A., Chakravarti A., Chen P.E., Cutler D.J., Kashuk C.S., Donnelly P., Marchini J., McVean G.A.T., Myers S.R., Cardon L.R., Abecasis G.R., Morris A., Weir B.S., Mullikin J.C., Sherry S.T., Feolo M., Daly M.J., Schaffner S.F., Qiu R., Kent A., Dunston G.M., Kato K., Niikawa N., Knoppers B.M., Foster M.W., Clayton E.W., Wang V.O., Watkin J., Sodergren E., Weinstock G.M., Wilson R.K., Fulton L.L., Rogers J., Birren B.W., Han H., Wang H., Godbout M., Wallenburg J.C., L'Archevêque P., Bellemare G., Todani K., Fujita T., Tanaka S., Holden A.L., Lai E.H., Collins F.S., Brooks L.D., McEwen J.E., Guyer M.S., Jordan E., Peterson J.L., Spiegel J., Sung L.M., Zacharia L.F., Kennedy K., Dunn M.G., Seabrook R., Shillito M., Skene B., Stewart J.G., (chair) D.L.V., (co-chair) E.W.C., (co-chair) L.B.J., Cho M.K., Duster T., Jasperse M., Licinio J., Long J.C., Ossorio P.N., Spallone P., Terry S.F., (chair) E.S.L., (co-chair) E.H.L., (co-chair) D.A.N., Boehnke M., Douglas J.A., Hudson R.R., Kruglyak L. & Nussbaum R.L. (2003) The International HapMap Project. *Nature* **426**, 789-96.

Gizaw S., Getachew T., Goshme S., Mwai O. & Dessie T. (2013) A cooperative village breeding scheme for smallholder sheep farming systems in Ethiopia. 5689.

Gravel S., Henn B.M., Gutenkunst R.N., Indap A.R., Marth G.T., Clark A.G., Yu F., Gibbs R.A., Project T.G., Bustamante C.D., Altshuler D.L., Durbin R.M., Abecasis G.R., Bentley D.R., Chakravarti A., Clark A.G., Collins F.S., Vega F.M.D.L., Donnelly P., Egholm M., Flicek P., Gabriel S.B., Gibbs R.A., Knoppers B.M., Lander E.S., Lehrach H., Mardis E.R., McVean G.A., Nickerson D.A., Peltonen L., Schafer A.J., Sherry S.T., Wang J., Wilson R.K., Gibbs R.A., Deiros D., Metzker M., Muzny D., Reid J., Wheeler D., Wang J., Li J., Jian M., Li G., Li R., Liang H., Tian G., Wang B., Wang J., Wang W., Yang H., Zhang X., Zheng H., Lander E.S., Altshuler D.L., Ambrogio L., Bloom T., Cibulskis K., Fennell T.J., Gabriel S.B., Jaffe D.B., Shefler E., Sougnez C.L., Bentley D.R., Gormley N., Humphray S., Kingsbury Z., Koko-Gonzales P., Stone J., McKernan K.J., Costa G.L., Ichikawa J.K., Lee C.C., Sudbrak R., Lehrach H., Borodina T.A., Dahl A., Davydov A.N., Marquardt P., Mertes F., Nietfeld W., Rosenstiel P., Schreiber S., Soldatov A.V., Timmermann B., Tolzmann M., Egholm M., Affourtit J., Ashworth D., Attiya S., Bachorski M., Buglione E., Burke A., Caprio A., Celone C., Clark S., Conners D., Desany B., Gu L., Guccione L., Kao K., Kebbel A., Knowlton J., Labrecque M., McDade L., Mealmaker C., Minderman M., Nawrocki A., Niazi F., Pareja K., Ramenani R., Riches D., Song W., Turcotte C., Wang S., Mardis E.R., Wilson R.K., Dooling D., Fulton L., Fulton R., Weinstock G., Durbin R.M., Burton J., Carter D.M., Churcher C., Coffey A., Cox A., Palotie A., Quail M., Skelly T., Stalker J., Swerdlow H.P., Turner D., Witte A.D., Giles S., Gibbs R.A., Wheeler D., Bainbridge M., Challis D., Sabo A., Yu F., Yu J., Wang J., Fang X., Guo X., Li R., Li Y., Luo R., Tai S., Wu H., Zheng H., Zheng X., Zhou Y., Li G., Wang J., Yang H., Marth G.T., Garrison E.P., Huang W., Indap A., Kural D., Lee W.-P., Leong W.F., Quinlan A.R., Stewart C., Stromberg M.P., Ward A.N., Wu

J., Lee C., Mills R.E., Shi X., Daly M.J., DePristo M.A., Altshuler D.L., Ball A.D., Banks E., Bloom T., Browning B.L., Cibulskis K., Fennell T.J., Garimella K.V., Grossman S.R., Handsaker R.E., Hanna M., Hartl C., Jaffe D.B., Kernytsky A.M., Korn J.M., Li H., Maguire J.R., McCarroll S.A., McKenna A., Nemesh J.C., Philippakis A.A., Poplin R.E., Price A., Rivas M.A., Sabeti P.C., Schaffner S.F., Shefler E., Shlyakhter I.A., Cooper D.N., Ball E.V., Mort M., Phillips A.D., Stenson P.D., Sebat J., Makarov V., Ye K., Yoon S.C., Bustamante C.D., Clark A.G., Boyko A., Degenhardt J., Gravel S., Gutenkunst R.N., Kaganovich M., Keinan A., Lacroute P., Ma X., Reynolds A., Clarke L., Flicek P., Cunningham F., Herrero J., Keenen S., Kulesha E., Leinonen R., McLaren W.M., Radhakrishnan R., Smith R.E., Zalunin V., Zheng-Bradley X., Korbel J.O., Stütz A.M., Humphray S., Bauer M., Cheetham R.K., Cox T., Eberle M., James T., Kahn S., Murray L., Chakravarti A., Ye K., Vega F.M.D.L., Fu Y., Hyland F.C.L., Manning J.M., McLaughlin S.F., Peckham H.E., Sakarya O., Sun Y.A., Tsung E.F., Batzer M.A., Konkel M.K., Walker J.A., Sudbrak R., Albrecht M.W., Amstislavskiy V.S., Herwig R., Parkhomchuk D.V., Sherry S.T., Agarwala R., Khouri H.M., Morgulis A.O., Paschall J.E., Phan L.D., Rotmistrovsky K.E., Sanders R.D., Shumway M.F., Xiao C., McVean G.A., Auton A., Iqbal Z., Lunter G., Marchini J.L., Moutsianas L., Myers S., Tumian A., Desany B., Knight J., Winer R., Craig D.W., Beckstrom-Sternberg S.M., Christoforides A., Kurdoglu A.A., Pearson J.V., Sinari S.A., Tembe W.D., Haussler D., Hinrichs A.S., Katzman S.J., Kern A., Kuhn R.M., Przeworski M., Hernandez R.D., Howie B., Kelley J.L., Melton S.C., Abecasis G.R., Li Y., Anderson P., Blackwell T., Chen W., Cookson W.O., Ding J., Kang H.M., Lathrop M., Liang L., Moffatt M.F., Scheet P., Sidore C., Snyder M., Zhan X., Zöllner S., Awadalla P., Casals F., Idaghdour Y., Keebler J., Stone E.A., Zilversmit M., Jorde L., Xing J., Eichler E.E., Aksay G., Alkan C., Hajirasouliha I., Hormozdiari F., Kidd J.M., Sahinalp S.C., Sudmant P.H., Mardis E.R., Chen K., Chinwalla A., Ding L., Koboldt D.C., McLellan M.D., Dooling D., Weinstock G., Wallis J.W., Wendl M.C., Zhang Q., Durbin R.M., Albers C.A., Ayub Q., Balasubramaniam S., Barrett J.C., Carter D.M., Chen Y., Conrad D.F., Danecek P., Dermitzakis E.T., Hu M., Huang N., Hurles M.E., Jin H., Jostins L., Keane T.M., Le S.Q., Lindsay S., Long Q., MacArthur D.G., Montgomery S.B., Parts L., Stalker J., Tyler-Smith C., Walter K., Zhang Y., Gerstein M.B., Snyder M., Abyzov A., Balasubramanian S., Bjornson R., Du

J., Grubert F., Habegger L., Haraksingh R., Jee J., Khurana E., Lam H.Y.K., Leng J., Mu X.J., Urban A.E., Zhang Z., Li Y., Luo R., Marth G.T., Garrison E.P., Kural D., Quinlan A.R., Stewart C., Stromberg M.P., Ward A.N., Wu J., Lee C., Mills R.E., Shi X., McCarroll S.A., Banks E., DePristo M.A., Handsaker R.E., Hartl C., Korn J.M., Li H., Nemesh J.C., Sebat J., Makarov V., Ye K., Yoon S.C., Degenhardt J., Kaganovich M., Clarke L., Smith R.E., Zheng-Bradley X., Korbel J.O., Humphray S., Cheetham R.K., Eberle M., Kahn S., Murray L., Ye K., Vega F.M.D.L., Fu Y., Peckham H.E., Sun Y.A., Batzer M.A., Konkel M.K., Walker J.A., Xiao C., Iqbal Z., Desany B., Blackwell T., Snyder M., Xing J., Eichler E.E., Aksay G., Alkan C., Hajirasouliha I., Hormozdiari F., Kidd J.M., Chen K., Chinwalla A., Ding L., McLellan M.D., Wallis J.W., Hurles M.E., Conrad D.F., Walter K., Zhang Y., Gerstein M.B., Snyder M., Abyzov A., Du J., Grubert F., Haraksingh R., Jee J., Khurana E., Lam H.Y.K., Leng J., Mu X.J., Urban A.E., Zhang Z., Gibbs R.A., Bainbridge M., Challis D., Coafra C., Dinh H., Kovar C., Lee S., Muzny D., Nazareth L., Reid J., Sabo A., Yu F., Yu J., Marth G.T., Garrison E.P., Indap A., Leong W.F., Quinlan A.R., Stewart C., Ward A.N., Wu J., Cibulskis K., Fennell T.J., Gabriel S.B., Garimella K.V., Hartl C., Shefler E., Sougnez C.L., Wilkinson J., Clark A.G., Gravel S., Grubert F., Clarke L., Flicek P., Smith R.E., Zheng-Bradley X., Sherry S.T., Khouri H.M., Paschall J.E., Shumway M.F., Xiao C., McVean G.A., Katzman S.J., Abecasis G.R., Blackwell T., Mardis E.R., Dooling D., Fulton L., Fulton R., Koboldt D.C., Durbin R.M., Balasubramaniam S., Coffey A., Keane T.M., MacArthur D.G., Palotie A., Scott C., Stalker J., Tyler-Smith C., Gerstein M.B., Balasubramanian S., Chakravarti A., Knoppers B.M., Abecasis G.R., Bustamante C.D., Gharani N., Gibbs R.A., Jorde L., Kaye J.S., Kent A., Li T., McGuire A.L., McVean G.A., Ossorio P.N., Rotimi C.N., Su Y., Toji L.H., TylerSmith C., Brooks L.D., Felsenfeld A.L., McEwen J.E., Abdallah A., Juenger C.R., Clemm N.C., Collins F.S., Duncanson A., Green E.D., Guyer M.S., Peterson J.L., Schafer A.J., Abecasis G.R., Altshuler D.L., Auton A., Brooks L.D., Durbin R.M., Gibbs R.A., Hurles M.E. & McVean G.A. (2011) Demographic history and rare allele sharing among human populations.

Ifergan I., Kebir H., Terouz S., Alvarez J.I., Lecuyer M.A., Gendron S., Bourbonniere L., Dunay I.R., Bouthillier A., Moumdjian R., Fontana A., Haqqani A., Klopstein A., Prinz M., Lopez-Vales R., Birchler T. & Prat A. (2011) Role of Ninjurin-1 in the migration of myeloid cells to central nervous system inflammatory lesions. *Ann Neurol* **70**, 751-63.

Jun J., Cho Y., Hu H., Kim H.-M., Jho S., Gadhvi P., Park K., Lim J., Paek W., Han K., Manica A., Edwards J.S. & Bhak J. (2014) Whole genome sequence and analysis of the Marwari horse breed and its genetic origin. *BMC Genomics* **15**, S4-S.

Klein Isaac A., Resch W., Jankovic M., Oliveira T., Yamane A., Nakahashi H., Di Virgilio M., Bothmer A., Nussenzweig A., Robbiani Davide F., Casellas R. & Nussenzweig Michel C. (2011) Translocation-Capture Sequencing Reveals the Extent and Nature of Chromosomal Rearrangements in B Lymphocytes. *Cell* **147**, 95-106.

Knezevic V., De Santo R., Schughart K., Huffstadt U., Chiang C., Mahon K.A. & Mackem S. (1997) Hoxd-12 differentially affects preaxial and postaxial chondrogenic branches in the limb and regulates Sonic hedgehog in a positive feedback loop. *Development* **124**, 4523-36.

Kolaczkowski B., Kern A.D., Holloway A.K. & Begun D.J. (2011) Genomic differentiation between temperate and tropical Australian populations of Drosophila melanogaster. *Genetics* **187**, 245-60.

Krzywinski M., Schein J., Birol I., Connors J., Gascoyne R., Horsman D., Jones S.J. & Marra M.A. (2009) Circos: an information aesthetic for comparative genomics. *Genome Res* **19**, 1639-45.

Li H. & Durbin R. (2009) Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics (Oxford, England)* **25**, 1754-60.

Lieberman-Aiden E., Berkum N.L.v., Williams L., Imakaev M., Ragoczy T., Telling A., Amit I., Lajoie B.R., Sabo P.J., Dorschner M.O., Sandstrom R., Bernstein B., Bender M.A., Groudine M., Gnirke A., Stamatoyannopoulos J., Mirny L.A., Lander E.S. & Dekker J. (2009) Comprehensive Mapping of Long-Range Interactions Reveals Folding Principles of the Human Genome.

Locke M.M., Penedo M.C.T., Bricker S.J., Millon L.V. & Murray J.D. (2002) Linkage of the grey coat colour locus to microsatellites on horse chromosome 25. *Animal genetics* **33**, 329-37.

Makvandi-Nejad S., Hoffman G.E., Allen J.J., Chu E., Gu E., Chandler A.M., Loredo A.I., Bellone R.R., Mezey J.G., Brooks S.a. & Sutter N.B. (2012) Four loci explain 83% of size variation in the horse. *PLoS ONE* **7**, e39929-e.

Manske M., Miotto O., Campino S., Auburn S., Almagro-Garcia J., Maslen G., O'Brien J., Djimde A., Doumbo O., Zongo I., Ouedraogo J.-B., Michon P., Mueller I., Siba P., Nzila A., Borrmann S., Kiara S.M., Marsh K., Jiang H., Su X.-Z., Amaratunga C., Fairhurst R., Socheat D., Nosten F., Imwong M., White N.J., Sanders M., Anastasi E., Alcock D., Drury E., Oyola S., Quail M.A., Turner D.J., Ruano-Rubio V., Jyothi D., Amenga-Etego L., Hubbart C., Jeffreys A., Rowlands K., Sutherland C., Roper C., Mangano V., Modiano D., Tan J.C., Ferdig M.T., Amambua-Ngwa A., Conway D.J., Takala-Harrison S., Plowe C.V., Rayner J.C., Rockett K.A., Clark T.G., Newbold C.I., Berriman M., MacInnis B. & Kwiatkowski D.P. (2012) Analysis of Plasmodium falciparum diversity in natural infections by deep sequencing. *Nature* **487**, 375-9.

McDonald R.E., Fleming R.I., Beeley J.G., Bovell D.L., Lu J.R., Zhao X., Cooper A. & Kennedy M.W. (2009) Latherin: A Surfactant Protein of Horse Sweat and Saliva. *Plos One* **4**, e5726.

Mi H., Muruganujan A., Casagrande J.T. & Thomas P.D. (2013) Large-scale gene function analysis with the PANTHER classification system. *Nature protocols* **8**, 1551-66.

Mills R.E., Luttig C.T., Larkins C.E., Beauchamp A., Tsui C., Pittard W.S. & Devine S.E. (2006) An initial map of insertion and deletion (INDEL) variation in the human genome. *Genome Res* **16**, 1182-90.

Mullaney J.M., Mills R.E., Pittard W.S. & Devine S.E. (2010) Small insertions and deletions (INDELs) in human genomes.

Nei M. & Li W.H. (1979) Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proc Natl Acad Sci U S A* **76**, 5269-73.

Nguyen D.Q., Webber C. & Ponting C.P. (2006) Bias of selection on human copy-number variants. *PLoS Genet* **2**, e20.

Orlando L., Ginolhac A., Zhang G., Froese D., Albrechtsen A., Stiller M., Schubert M., Cappellini E., Petersen B., Moltke I., Johnson P.L.F., Fumagalli M., Vilstrup J.T., Raghavan M., Korneliussen T., Malaspinas A.-S., Vogt J., Szklarczyk D., Kelstrup C.D., Vinther J., Dolocan A., Stenderup J., Velazquez A.M.V., Cahill J., Rasmussen M., Wang X., Min J., Zazula G.D., Seguin-Orlando A., Mortensen C., Magnussen K., Thompson J.F., Weinstock J., Gregersen K., Røed K.H., Eisenmann V., Rubin C.J., Miller D.C., Antczak D.F., Bertelsen M.F., Brunak S., Al-Rasheid K.A.S., Ryder O., Andersson L., Mundy J., Krogh A., Gilbert M.T.P., Kjær K., Sicheritz-Ponten T., Jensen L.J., Olsen J.V., Hofreiter M., Nielsen R., Shapiro B., Wang J. & Willerslev E. (2013) Recalibrating Equus evolution using the genome sequence of an early Middle Pleistocene horse. *Nature* **499**, 74-8.

Petersen J.L., Mickelson J.R., Rendahl A.K., Valberg S.J., Andersson L.S., Axelsson J., Bailey E., Bannasch D., Binns M.M., Borges A.S., Brama P., da Câmara Machado A., Capomaccio S., Cappelli K., Cothran E.G., Distl O., Fox-Clipsham L., Graves K.T., Guérin G., Haase B., Hasegawa T., Hemmann K., Hill E.W., Leeb T., Lindgren G., Lohi H., Lopes M.S., McGivney B.a., Mikko S., Orr N., Penedo M.C.T., Piercy R.J., Raekallio M., Rieder S., Røed K.H., Swinburne J., Tozaki T., Vaudin M., Wade C.M. & McCue

M.E. (2013) Genome-wide analysis reveals selection for important traits in domestic horse breeds. *PLoS genetics* **9**, e1003211-e.

Pitera J.E., Milla P.J., Scambler P. & Adjaye J. (2001) Cloning of HOXD1 from unfertilised human oocytes and expression analyses during murine oogenesis and embryogenesis. *Mechanisms of Development* **109**, 377-81.

Quach H., Barreiro L.B., Laval G., Zidane N., Patin E., Kidd K.K., Kidd J.R., Bouchier C., Veuille M., Antoniewski C. & Quintana-Murci L. (2009) Signatures of Purifying and Local Positive Selection in Human miRNAs. *The American Journal of Human Genetics* **84**, 316-27.

Schubert M., Jónsson H., Chang D., Sarkissian C.D., Ermini L., Ginolhac A., Albrechtsen A., Dupanloup I., Foucal A., Petersen B., Fumagalli M., Raghavan M., Seguin-Orlando A., Korneliussen T.S., Velazquez A.M.V., Stenderup J., Hoover C.A., Rubin C.-J., Alfarhan A.H., Alquraishi S.A., Al-Rasheid K.A.S., MacHugh D.E., Kalbfleisch T., MacLeod J.N., Rubin E.M., Sicheritz-Ponten T., Andersson L., Hofreiter M., Marques-Bonet T., Gilbert M.T.P., Nielsen R., Excoffier L., Willerslev E., Shapiro B. & Orlando L. (2014) Prehistoric genomes reveal the genetic foundation and cost of horse domestication.

Signer-Hasler H., Flury C., Haase B., Burger D., Simianer H., Leeb T. & Rieder S. (2012) A Genome-Wide Association Study Reveals Loci Influencing Height and Other Conformation Traits in Horses. *Plos One* **7**, e37282.

Thomas J.W., Touchman J.W., Blakesley R.W., Bouffard G.G., Beckstrom-Sternberg S.M., Margulies E.H., Blanchette M., Siepel A.C., Thomas P.J., McDowell J.C., Maskeri B., Hansen N.F., Schwartz M.S., Weber R.J., Kent W.J., Karolchik D., Bruen T.C., Bevan R., Cutler D.J., Schwartz S., Elnitski L., Idol J.R., Prasad A.B., Lee-Lin S.Q., Maduro V.V., Summers T.J., Portnoy M.E., Dietrich N.L., Akhter N., Ayele K., Benjamin B., Cariaga K., Brinkley C.P., Brooks S.Y., Granite S., Guan X., Gupta J., Haghighi P., Ho S.L., Huang M.C., Karlins E., Laric P.L., Legaspi R., Lim M.J., Maduro Q.L., Masiello

C.A., Mastrian S.D., McCloskey J.C., Pearson R., Stantripop S., Tiongson E.E., Tran J.T., Tsurgeon C., Vogt J.L., Walker M.A., Wetherby K.D., Wiggins L.S., Young A.C., Zhang L.H., Osoegawa K., Zhu B., Zhao B., Shu C.L., De Jong P.J., Lawrence C.E., Smit A.F., Chakravarti A., Haussler D., Green P., Miller W. & Green E.D. (2003) Comparative analyses of multi-species sequences from targeted genomic regions. *Nature* **424**, 788-93.

Untergasser A., Cutcutache I., Koressaar T., Ye J., Faircloth B.C., Remm M. & Rozen S.G. (2012) Primer3--new capabilities and interfaces. *Nucleic Acids Res* **40**, e115.

Van der Auwera G.A., Carneiro M.O., Hartl C., Poplin R., del Angel G., Levy-Moonshine A., Jordan T., Shakir K., Roazen D., Thibault J., Banks E., Garimella K.V., Altshuler D., Gabriel S. & DePristo M.A. (2013) From fastQ data to high-confidence variant calls: The genome analysis toolkit best practices pipeline. *Current Protocols in Bioinformatics*.

Vance S.J., McDonald R.E., Cooper A., Smith B.O. & Kennedy M.W. (2013) The structure of latherin, a surfactant allergen protein from horse sweat and saliva.

Wade C.M., Giulotto E., Sigurdsson S., Zoli M., Gnerre S., Imsland F., Lear T.L., Adelson D.L., Bailey E., Bellone R.R., Blöcker H., Distl O., Edgar R.C., Garber M., Leeb T., Mauceli E., MacLeod J.N., Penedo M.C.T., Raison J.M., Sharpe T., Vogel J., Andersson L., Antczak D.F., Biagi T., Binns M.M., Chowdhary B.P., Coleman S.J., Della Valle G., Fryc S., Guérin G., Hasegawa T., Hill E.W., Jurka J., Kiialainen a., Lindgren G., Liu J., Magnani E., Mickelson J.R., Murray J., Nergadze S.G., Onofrio R., Pedroni S., Piras M.F., Raudsepp T., Rocchi M., Røed K.H., Ryder O.a., Searle S., Skow L., Swinburne J.E., Syvänen a.C., Tozaki T., Valberg S.J., Vaudin M., White J.R., Zody M.C., Lander E.S. & Lindblad-Toh K. (2009) Genome sequence, comparative analysis, and population genetics of the domestic horse. *Science (New York, N.Y.)* **326**, 865-7.

Wang W., Wang S., Hou C., Xing Y., Cao J., Wu K., Liu C., Zhang D., Zhang L., Zhang Y. & Zhou H. (2014) Genome-Wide Detection of Copy Number Variations among Diverse Horse Breeds by Array CGH. *Plos One* **9**, e86860.

Warmuth V., UK U.o.C.D.o.Z.C., Manica A., UK U.o.C.D.o.Z.C., Eriksson A., UK U.o.C.D.o.Z.C., Barker G., UK U.o.C.M.I.f.A.R.C., Bower M. & UK U.o.C.M.I.f.A.R.C. (2015) Autosomal genetic diversity in non-breed horses from eastern Eurasia provides insights into historical population movements. *Animal genetics* **44**, 53-61.

Womack J.E. (2005) Advances in livestock genomics: Opening the barn door.

Yu N., Jensen-Seaman M.I., Chemnick L., Ryder O. & Li W.-H. (2004) Nucleotide Diversity in Gorillas.

Zeitouni B., Boeva V., Janoueix-Lerosey I., Loeillet S., Legoix-né P., Nicolas A., Delattre O. & Barillot E. (2010) SVDetect: a tool to identify genomic structural variations from paired-end and mate-pair sequencing data. *Bioinformatics (Oxford, England)* **26**, 1895-6.

Zhao Z., Fu Y.-X., Hewett-Emmett D. & Boerwinkle E. (2003) Investigating single nucleotide polymorphism (SNP) density in the human genome and its implications for molecular evolution. *Gene* **312**, 207-13.

CHAPTER 3

GENOME-WIDE SCANS REVEAL QTLs FOR WITHERS HEIGHT IN HORSES NEAR *ANKRD1* AND *IGF2BP2*

Mohammed A. Al Abri[1,2], Christian Posbergh[1], Nathan B. Sutter[3], John Eberth[4], Gabriel E. Hoffman[5], and Samantha A. Brooks[1]*

[1]Department of Animal Science, 149 Morrison Building, Cornell University, Ithaca, NY 14853, USA

[2]Department of Animal and Veterinary Sciences, College of Agriculture and Marine Sciences, Sultan Qaboos University, PO box 34 Al Khod, Postal Code 123, Muscat, Oman

[3]Department of Biology, La Sierra University, 4500 Riverwalk Parkway, Riverside, CA 9251

[4]University of Kentucky, 900 W. P. Garrigus Building, Lexington, KY 40546

[5]Mount Sinai Hospital, Icahn Medical Institute, Floor 3 Room L3-70, Desk 26, 1425 Madison Avenue, New York, NY 10029

*Corresponding author

*Now Department of Animal Sciences, University of Florida, Gainesville, FL 32611, USA

ABSTRACT

Withers height is an important trait for the American Miniature horses since the breed objective is to produce the small and proportionate animals. Accordingly, the breed registry dictates that the height of the mature Miniature horse at the withers must not exceed 34 inches (0.864 meters) (American Miniature Horse Association Accessed April 28, 2014). Therefore, identification of Quantitative Trait Loci (QTLs) affecting this trait will result in a better understanding of the genetic architecture and biological pathways contributing to skeletal height in the horse. Using the Equine SNP50 bead chip (Illumina Inc), we previously genotyped 48 horses from 16 different breeds that represent extremes in body size (Makvandi-Nejad *et al.* 2012). We applied a dominant model Genome Wide Association Study (GWAS) and found loci affecting size variation in the horse that were not previously reported. We complemented our GWAS findings by conducting a genome wide F_{ST} estimation as well as a cross-population composite likelihood ratio test (XP-CLR) test between the eight large and eight small breeds. The ECA1: 37676322 bp markers, positioned within an intron of the *ANKRD1* gene, were detected by both the GWAS and the XP-CLR scan. To confirm our findings at this locus, we used a PCR–Restriction Fragment Length Polymorphism (PCR-RFLP) to genotype 90 additional American Miniature horses. Within this population we verified that ECA1: 37676322 bp marker indeed follows a dominant mode of inheritance. Horses possessing the GG or AG genotypes were 4.064 cm (1.6 inches) taller on average than horses with the AA genotype. *ANKRD1* is a transcription factor that is involved in muscle myocytes and cardiomyocyte growth and differentiation and may contribute to height by influencing the overall growth of the horses. This marker will be a valuable tool for selection of breeding stock in breeds with height restrictions for registration.

INTRODUCTION

Horses have been selected for generations for diverse skeletal compositions in order to perform various tasks (Brooks *et al.* 2010b). As a result of that selection, they now have ample skeletal size variability both within and between breeds. Skeletal size in the horse is highly heritable with an overall mean heritability of 0.49 ± 0.065 (Saastamoinen (1990). This means that 49 % of the variation in horse height is due to additive genetic inheritance. The genetic architecture of horse size variation seems to be controlled by a few genes due to intense selection for size. Makvandi-Nejad *et al.* (2012) found 4 loci that together explained 83% in horse size variation. In contrast, height variation in humans is controlled by over 600 loci and only 36% of the variability could be explained by these loci (Wood *et al.* 2014). Withers height was used successfully to map QTLs involved in height variation in horses close to *LCORL/NCAPG* genes in chromosome 3 and *ZFAT* gene in chromosome 9 (Signer-Hasler *et al.* 2012). However, the first principal component (PC1), created by pooling 33 body measurements, is a comprehensive, quantitative trait that explained the majority (65.9%) of skeletal size variation in horses (Makvandi-Nejad *et al.* 2012).

Using genome-wide association study (GWAS) has aided researchers to identify biologically meaningful candidate genes close to the significant SNPs. The availability of a high-density SNP chip for the horse, helped conducting GWAS that resulted in mapping QTLs at a very fine-scale (Brooks *et al.* 2010a). Mixed model GWAS greatly reduces the population structure, and therefore false positive results (Shin & Lee 2015), which narrows down the regions of the genome that are more likely to contain the causative mutations. Therefore, they are used extensively in studies involving admixed or related groups of individuals e.g (Sutter *et al.* 2007) and (Guo *et al.* 2012). SNP chip genotypes can also be used in screening the genome for regions containing signatures of selection. This lead to the discovery of various biologically relevant genes in cattle (Pérez O'Brien *et al.* 2014), pig (Rubin *et al.* 2012) and sheep (McRae *et al.* 2014).

The aim of this study was to identify QTLs controlling size variation in withers height in the horse. We used withers height as a proxy to measure skeletal size phenotype as it is easy to measure and is highly correlated with PC1 (r=0.93, p<0.005). Instead of the standard additive model GWAS, we used dominance and a recessive mixed model GWAS. Advantageously, our data set was comprised of diverse breeds representing the extremes in skeletal morphology in an equal proportion. We therefore decided to conduct a genome-wide search for regions harboring signatures of selection using two methods by splitting the animals into two groups of large and small breeds. The first method was the F_{ST} genetic differentiation test to search (Weir & Cockerham 1984). The second method is called the cross-population composite likelihood ratio test (XP-CLR) test which is based on the multi-locus allele frequency differentiation between two populations (Chen *et al.* 2010). Both the F_{ST} and XP-CLR utilize the variation in allele frequencies between two populations to detect selective sweeps. We found that, using a dominance model, we could detect loci that were not previously reported. Using both, GWAS and XP-CLR, we detected that *ANKRD1* is significantly associated with withers height variation in the horse.

MATERIALS AND METHODS

Animal resources, samples collection and genotyping

The trait withers height was defined following Brooks *et al.* (2010b), as the measure from the ground to the highest point of the withers as shown in **Figure 3.1**. Samples and measurements were taken from horses of volunteering horse owners. In total, there were 48 horses belonging to

16 different breeds (3 from each breed) used in this study (**Table 3.1**). Horses were measured either by their owners or by laboratory staff and collaborators. The breed identity and animal age were provided by the owner and confirmed by examining the photo of each animal. DNA was collected either from whole blood or tail hair bulbs using standard methods as previously described in (Cook *et al.* 2010). Genotyping was performed using the equine SNP 50 Illumina BeadChip (GeneSeek, Lincoln, NE, USA).

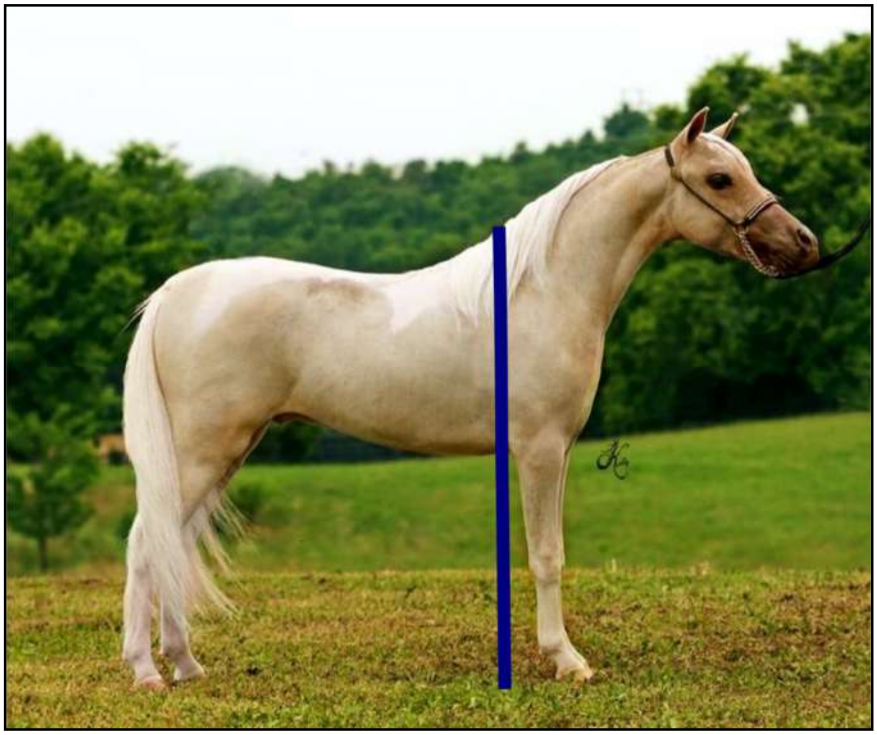


Figure 3.1. Illustration of the withers height phenotype shown as the measure from the ground to the highest point of the withers.

Table 3.1. Three horses of each of the following horse breeds have been genotyped for the study. The breed name and mean withers height for each breed in cm are given.

Breed	Mean withers Height
American Belgian	171.0
American Miniature	77.6
Ardennais	155.4
Brabant	168.9
Caspian	116.4
Clydesdale	179.5
Dartmoor Pony	125.3
Falabella	87.6
Friesian	163.8
Percheron	177.0
Puerto Rican Paso Fino	133.8
Shetland Pony	109.5
Shire	190.9
Suffolk Punch	165.1
Welsh Mountain Pony	119.8
Welsh Pony	126.4

SNPs quality control

SNPs with more than 20% missingness rate or those with a minor allele frequency less than 10% were filtered out of the dataset. Of the initial set of 54,624 SNPs, 16,938 SNPs were removed due to low minor allele frequency and 505 SNPs (0.92%) were removed due genotype missingness, leaving 37,584 SNPs for analysis. We also tested for samples duplicates using identity by state (IBS) check in PLINK (Purcell *et al.* 2007) and detected no sample duplicates.

Statistical analyses for the GWAS

We first performed the GWAS analysis using a standard dominance model association (with gender as a covariate) using the option --dominant in PLINK. Later we ran the same analysis using EMMA (Kang *et al.* 2008) which, unlike the standard linear model, includes the kinship as a random effect in a mixed model approach. Using a mixed model for the analysis allowed us to account for the population structure and therefore reducing false positive hits (Kang *et al.* 2008). Our model was as follows:

$$y = X\beta + Zu + e$$

Where **y** is the vector of phenotypic values of withers height in inches, X is a 48 by 3 matrix of fixed effects including the mean (μ) , SNPs, and gender. β is the fixed effects coefficients vector. Z is a design matrix that maps the phenotype to the corresponding breed. **u** is the random effect with Var (u) = σ_g^2 K, where K is the genomic kinship relationship matrix and **e** is the residual effect where e ~ N(0, I σ_e^2). Assuming that A and a be the major and minor alleles at a SNP respectively, our genotype coding (scores) for the dominant model was 0, 1, 1 for AA, Aa/Aa, aa. The genotype coding for the recessive model was 0, 0, 1 for AA, Aa/Aa, aa.

Genome-wide search for selective sweeps

First, the animals were separated into a large and a small breeds group. A cutoff of 60 inches (152 meters) was chosen to separate the animals into these small (24 animals) and large (24 animals) groups, based on the distribution of withers height measurements (**Figure 3.2)**. In order to search for regions with unusually strong polymorphism patterns genome wide (i.e regions of elevated population subdivision signals), the F_{ST} genetic differentiation test was calculated (Weir & Cockerham 1984). The F_{ST} test was performed using the R package pegas (Paradis 2010). To search for regions were the change in allele frequency occurred quickly, perhaps as a result of random drift, we calculated the cross population XP-CLR scores using the script available at (http://genetics.med.harvard.edu/reich/Reich_Lab/Software.html). Our parameters were as follows: non-overlapping sliding windows of 50 kb with a maximum number of SNPs per window being 30 SNPs and a correlation level of 0.95 was required to down-weight the contribution of SNPs to XP-CLR. The top 1% values of the empirical distribution of F_{ST} and XP-CLR values were considered 'selection outliers', i.e suggestive of a positive and/or divergent selection.

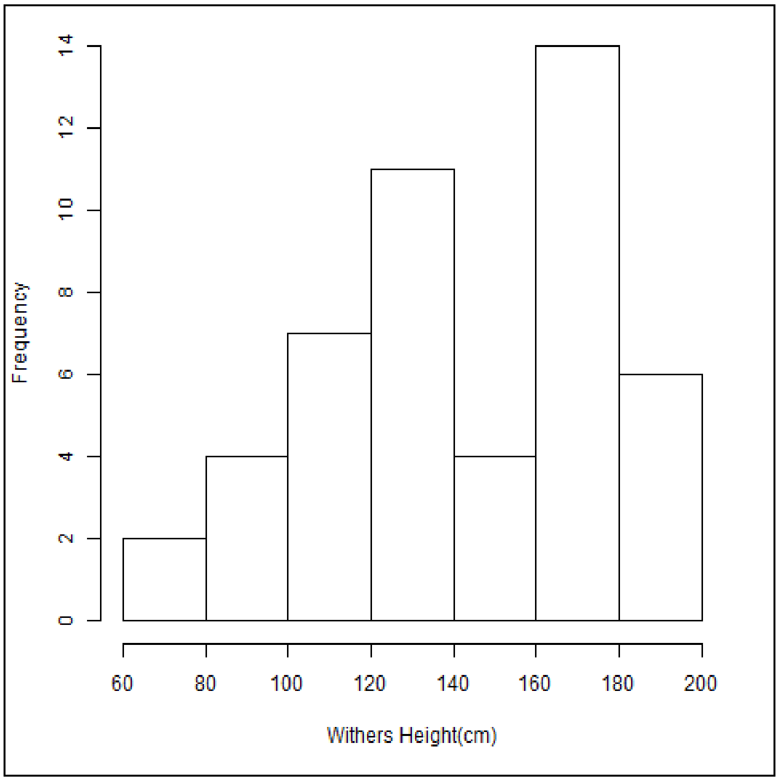


Figure 3.2. Horses for this study were selected to represent the extremes of skeletal size variation. Distribution of withers height in cm is shown in the figure. For the Fst and XP-CLR analysis a cut off of 152 cm was chosen representing the midpoint of the distribution.

Functional annotation of GWAS results and selective sweeps regions

Loci surpassing a genome wide significance level as well as genomic regions with "significant" selection signals identified by Fst and XP-CLR tests were annotated to the closest ENSEMBLE genes in EquCab2. Genes overlapping a span of 50 KB around significant selection signals positions were considered candidate genes. A span of 50 KB was used since it is the average LD length across breeds of horse (Wade *et al.* 2009).

PCR–RFLP detection

Genomic DNA was amplified for the *ANKRD1* locus by PCR using the primers: ANKRD1_F2 5'- GTC TGT GAC GAG GTA AGG CT – 3' and ANKRD1_R2 5' – GCC AAA TGT CCT TCC AAG CA – 3'. Reactions were made to a 20 µl volume using FastStart Taq DNA Polymerase and all necessary reagents according to the manufacturers recommended conditions (Roche Diagnostics, Indianapolis, IN). Thermocycling on an Eppendorf Mastercycler Ep Gradient (Eppendorf Corp., Westbury, NY) was also according to the manufacturer's recommendations with an annealing temperature of 58°C and a total of 40 cycles for this primer pair. The restriction digest used 10 µl PCR product, 0.5 U of Cac8I (New England Biolabs Inc. (NEB), Ipswitch, MA), 1x NEB Cutsmart Buffer, and enough MilliQ water to achieve a total volume of 20 µl per reaction, and were incubated at 37°C for two hours. The digest products were visualized following electrophoresis on a 2% agarose gel (Omnipur Agarose, EMD Chemicals Inc, Gibbstown, NJ). Agarose gels were stained (SYBRsafe DNA gel stain (10,000X) concentrate, Invitrogen Molecular Probes, Eugene, OR) and visualized under UV illumination (FluroChem HD2, Alpha Innotec Corp., San Leandro CA). The G allele resulting in fragments of 675, 402, and 20bp in size, while the A allele produced 1077 and 20bp fragments.

RESULTS AND DISCUSSION

Association analysis

The QQ plot of GWAS p-values for the linear model (PLINK) and the mixed model (EMMA), presented in **Figure 3.3**, shows clearly the EMMA correction for the population structure within this population. The genomic inflation factor (λ) after EMMA correction was 1.0695, which is suitable for GWAS. We detected no significant associations using the recessive model. Using the dominance model we detected 3 out of the 4 loci found by Makvandi-Nejad *et al.* 2012

(**Figure 3.4.a**). In addition, we detected two novel loci; the first locus was within an intron of the *Ankyrin Repeat Domain 1* (*ANKRD1*) gene at ECA1**:** 37676322. *ANKRD1* is a transcriptional factor that belongs to the muscle ankyrin repeat proteins (MARP) family, and encodes a cardiac ankyrin repeat protein (CARP) (Duboscq-Bidot *et al.* 2009). Of the members of the MARP protein family, *ANKRD1* (CARP) is mainly expressed in cardiac muscle. The other two members of the MARP family are the Ankyrin repeat domain protein 2 (Ankrd2/Arpp) is expressed in skeletal muscle, and the diabetes related ankyrin repeat protein (Ankrd23/ DARP) which is expressed at similar amounts in cardiac and skeletal muscle (Bang *et al.* 2014). During embryonic development, MARP proteins are expressed in developing skeletal muscles and are a vital for muscle morphogenesis (Baumeister *et al.* 1997). It is suggested that MARP as a nuclear cofactor is crucial in the signaling pathways starting with prospective tendon mesenchyme to forming muscle. It is also deemed to be involved in the signaling pathways from activated muscle interstitial cells to denervated muscle fibers (Baumeister *et al.* 1997). It is well established that the *ANKRD1* is upregulated in response to cardiac hypertrophy (Aihara *et al.* 2000). Missense mutations in *ANKRD1* were found to be causative of hypertrophic cardiomyopathy (Arimura *et al.* 2009). Nevertheless, Bang *et al.* (2014) showed that mice knock out for all three MARP genes (MKO mice) have normal heart morphology and function. MKO mice had an increased expression of the MyoD and Muscle LIM protein genes, suggestive of the role of the MARP proteins play in the expression regulation of muscle genes (Barash *et al.* 2007). *ANKRD1* itself is involved in the signaling pathways of muscle remodeling and

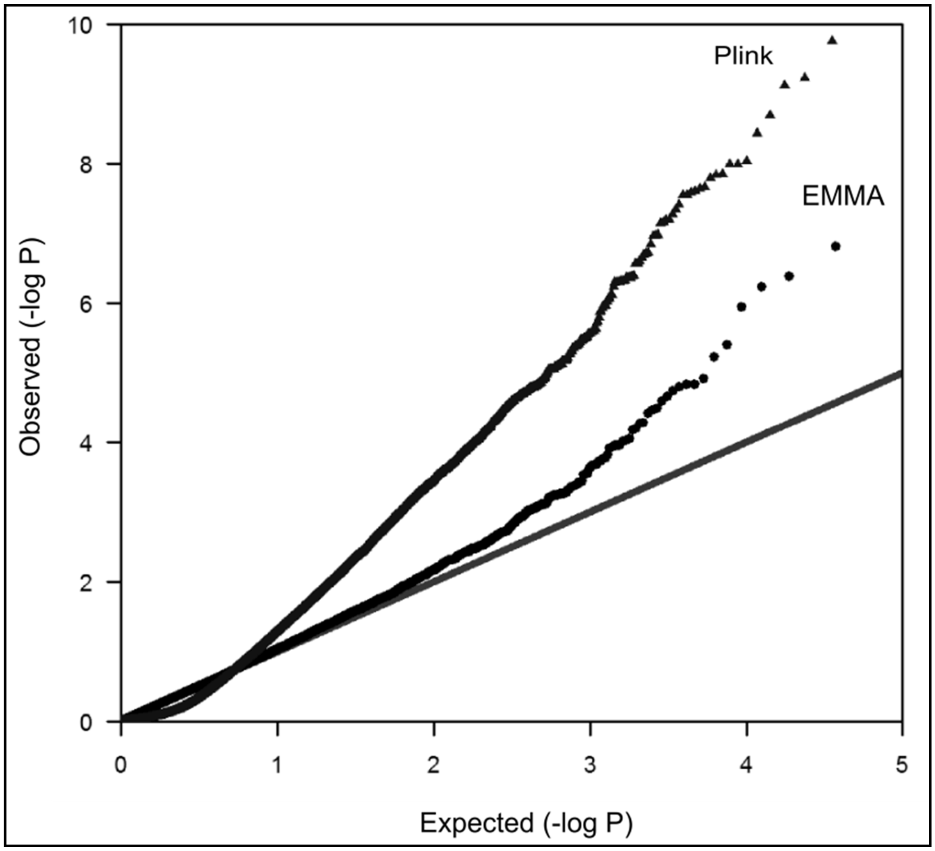


Figure 3.3 : The QQ plots for the GWAS analysis. The QQ-plot of the mixed model analysis using EMMA (solid circles) shows the considerable control of population structure (genomic inflation factor of 1.0695) compared to the standard linear model analysis in PLINK (solid triangles).

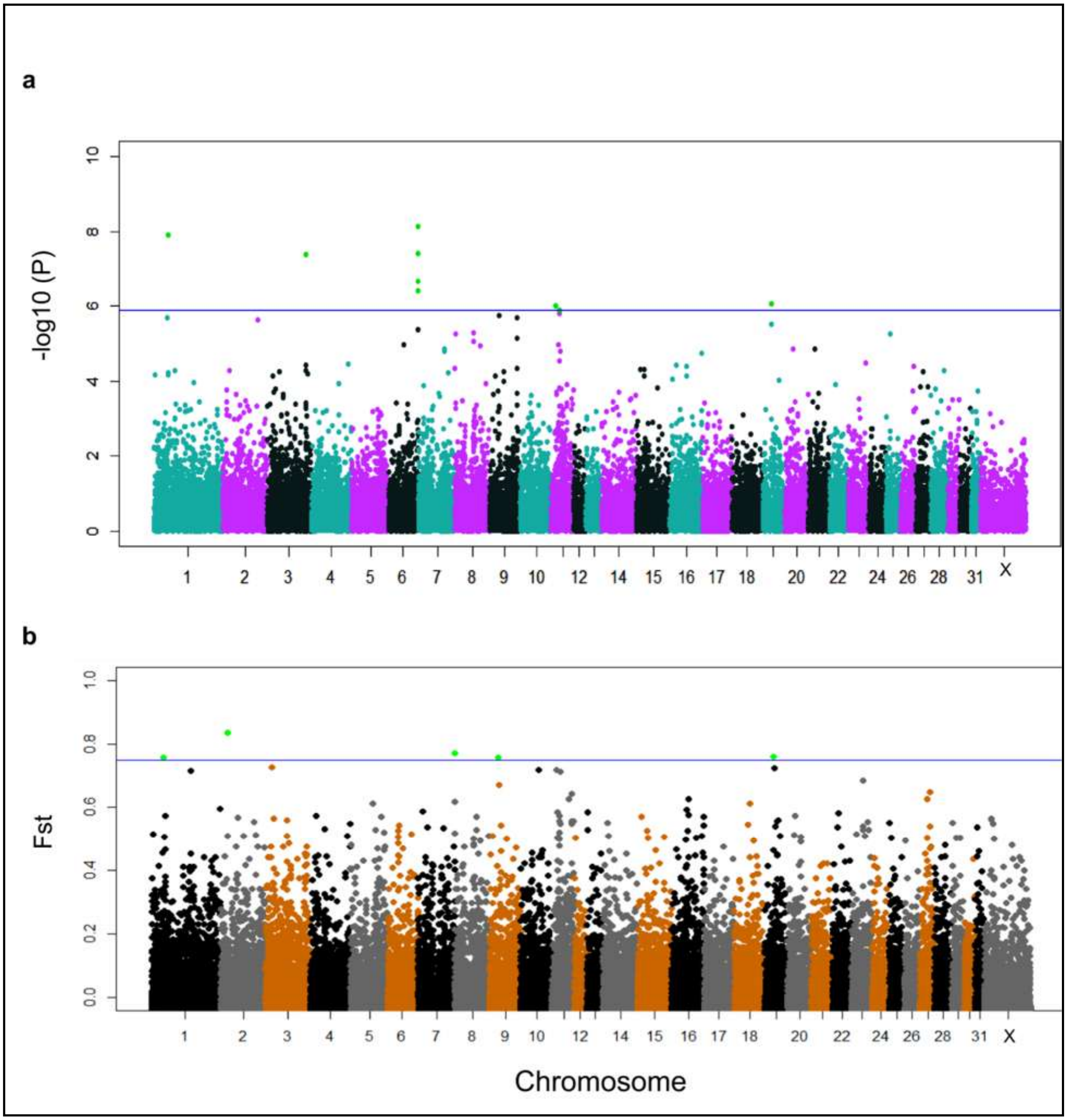


Figure 3.4: **a.** Manhattan plot of withers height of 48 horses from 16 breeds of extreme size based on a dominant model GWAS conducted in EMMA. The horizontal line indicates a genome-wide significance (alpha =0.05). **b.** Genome wide FST statistic values. The horizontal line at 0.75 is the significance level i.e line above which the highest 0.01 of the FST hits belong.

differentiation (Kojic *et al.* 2011). It was also found to be involved in wound healing through stimulating collagen gel contraction and actin fiber organization (Samaras *et al.* 2015). The second marker we identified as associated with height in the horse was within an intron in insulin-like growth factor 2 mRNA binding protein 2 (IGF2BP2) which is at ECA19: 23815750. *IGF2BP2* is best known for its role in insulin regulation (Groenewoud *et al.* 2008) and risk of type 2 diabetes (Saxena *et al.* 2007). It is a member of the conserved family of Insulin-like growth factor 2 mRNA-binding proteins family (*IGF2BP*) which also includes *IF2BP1* and *IGF2BP3*. The *IGF2BP* family is highly expressed in embryonic development, specifically in the period between zygote and embryo stages (Hansen *et al.* 2004). *IGF2BP1* and *IGF2BP3* are primarily expressed during embryonic development rather than in adult tissues (Bell *et al.* 2013). Although *IGF2BP2* is also expressed in embryonic development, it continues to be expressed in adult mice and human tissues such as the brain, muscles, kidney, liver and bone marrow (Christiansen *et al.* 2009). Targeted silencing of *HMGA2*, a gene previously implicated to affect overall size in horses (Makvandi-Nejad *et al.* 2012), causes the downregulation of *IGF2BP2* but not its family members *IGF2BP1* and *IGF2BP3* (Brants *et al.* 2004). *HMGA2*-deficient mice display the pygmy-phenotype and their *IGF2BP2* expression was below detectable levels which is suggestive of the role of *IGF2BP2* in embryogenesis, growth and development (Brants *et al.* 2004). The involvement of the HMGA2-IGF2BP2 axis in myoblast growth and overall development are supported by a similar but more recent experiment in which expression IGF2BP2 rescued the phenotype strongly supporting its involvement in growth (Li *et al.* 2012).

Identification of signals for selective sweeps

Loci bearing signatures of selection may elucidate the forces that helped shape an animal during evolution and domestication, and can facilitate the identification of variants that affect different morphologies. Scanning the genome for such signatures in domestic animals is particularly interesting as they harbor more phenotypic diversity than experimental organisms (Andersson *et al.* 2015). In a way, humans have conducted a long term genetic experiment in which they have altered the frequency of desired/undesired mutations in domesticated species to their advantage. The horse breeds used in this study have been selected for millennia for different skeletal types in order to meet specific tasks. Therefore, searching for selection signatures in such a set of extreme morphology for size can show how artificial selection has rapidly shaped this phenotype. These scans have previously shed light on the genomic regions affecting size variation in humans (Jarvis *et al.* 2012) and horses (Petersen *et al.* 2013b).

Numerous methods exist to search for selection signatures using genotypic data. These can be broadly divided into between populations and within population methods. Example of the within population methods include Tajima's D (Tajima 1989) and the integrated haplotype score (iHS) (Voight *et al.* 2006) and Compsite Likelihood Ratio Test (CLR) (Nielsen *et al.* 2005). Both Tjima's D and CLR test are based on the change on the allele frequency around a sweep whereas the iHS is based on LD patterns around the sweep. Here we utilized two different between populations methods to infer putative selective sweeps. The first was the F_{ST} statistic (Wright 1949) and is a more traditional method for that uses variation of allele frequency between two populations to detect selection footprints. Since its introduction, different flavors of it were developed such as the Fst-based Bayesian hierarchical model (Riebler *et al.* 2008) and the two-step Fst method (Gianola *et al.* 2010). The second method was the Cross Population Composite Likelihood Ratio (XP-CLR) (Chen *et al.* 2010) is composite likelihood method that uses an outgroup population to detect departures from neutrality which maybe be compatible with soft or hard sweeps close to the beneficial allele. Like F_{ST}, XP-CLR also utilizes the variation of allele frequency between two populations to identify signatures of selection. A possible downside to this method is that it is sensitive to recent selection and could miss selective events that occurred a long time ago (Ma *et al.* 2014).Other methods to detect selective sweeps by comparing two populations exist such as the Cross Population Extend Haplotype Homozygosity Test (XPEHH) which searches for the selection footprint around beneficial sites through assessing linkage disequlibrium (LD) patterns (Sabeti *et al.* 2007).

We identified four sweep regions using a genome-wide FST approach (**Figure 3.4.b**). The four markers suggestive of a recent selective sweep were within introns of the R3H domain and coiled-coil containing 1-like (*R3HCC1L*), Microtubule-Actin Crosslinking Factor 1 (*MACF1*), Calcineurin Binding Protein 1 (*CABIN1*), and *IGF2BP2* genes*. R3HCC1L,* is known to be involved in growth inhibition and differentiation (www.genecards.org). On the other hand, *MACF1* is involved in microtubule actin cross-linking development and mice with *MACF1* deletion *(*MACF1−/−**)** exhibit growth retardation (Chen *et al.* 2006). *CABIN1* was found to play a role in skeletal muscle development (Friday *et al.* 2000). Given that all these genes are involved in growth related functions, it is plausible that they may also exert an effect in skeletal size variation in horse. This is specially the case for the *IGF2BP2* locus which was also detected in the GWAS analysis. In addition, genes within a 50 KB window of significant F_{ST} locations (**Table 3.2**) include *D*-dopachrome tautomerase (*DDT*) and deSUMOylation (*SENP2*). *DDT* produces a cytokine that is up-regulated in Patients with Sepsis or Invasive Cancer (Merk *et al.* 2011) whereas *SENP2* is involved in liver cancer cell proliferation (Tu *et al.* 2015).

Genes within 50 KB of the XP-CLR sweeps are shown in **Table 3.2.** The four highest genome-wide XP-CLR scores were at the *ANKRD1* locus, providing further evidence for its role in growth and development of the horse. Amongst the loci within the top 1% of XP-CLR scores we also detected the High-mobility group AT-hook (*HMGA2)*. *HMGA2* is primarily expressed during embryonic development but is also found in human benign tumors of mesenchymal origin (Fedele *et al.* 2002). Over-expression of *HMGA2* leads to increased secretion prolactin/growth hormone leading to cell pituitary adenomas (Fedele *et al.* 2002).The product of the *HMGA2* gene is suggested to confer variation in growth through regulation of cellular proliferation (Young & Narita 2007). The gene was also previously reported in height GWAS studies of height in dogs (Boyko *et al.* 2010) and humans (Weedon *et al.* 2007). In humans, an overgrowth syndrome affected an individual who carried a chromosomal inversion truncating the HMGA2 (Ligon *et al.* 2005). In mice deletions of homolog of *IGF2BP2* result in dwarfism (Zhou *et al.* 1995) and the dwarf phenotype in chicken was mapped to syntenic region (Ruyter-Spira *et al.* 1998). As noted earlier, *HMGA2* is hypothesized to alter skeletal growth by directly regulating the *IGF2BP2* gene (Li *et al.* 2012).

Table 3.2. Genes within 50 KB of the GWAS, FST and XP-CLR significant regions. *ANKRD1* was detected using the XP-CLR and the GWAS, while *IGF2BP2* was detected using the GWAS and the FST scan.

XP-CLR	**GWAS**	**FST**
ANKRD1	ANKRD1	R3HCC1L
RPP30	RPP30	MACF1
HTR7	HTR7	CABIN1
HMGA2	HMGA2	DDT
ZAP70	IGF2BP2	IGF2BP2
TMEM131	SNORD61	SENP2
RUNX3	ECA-MIR-763	
CLIC4	ABCA9	
ACTR1B	ABCA8	
CDC42EP1	RPL23	
LGALS2	FBXO47	
ANKRD54	LASP1	
TRIOBP	SNORA21	
GCAT	C17ORF98	
PLCE1	CWC25	
HSPB9		
KAT2A		
DHX58		
ZNF385C		
NKIRAS2		
DNAJC7		
SEMA6A		
GORASP2		
TLK1		
ENOX1		
AGBL4		
LMO4		
TFEC		
MRPS6		
MICU3		
FGF20		

Validation of the *ANKRD1* locus in a second population of animals

We chose markers near *ANKRD1* for subsequent validation as these possessed the highest XP-CLR score and the smallest p-value in the GWAS. We used a custom PCR-RFLP assay to genotype a total of 90 American miniature horses at for the A>G SNP at ECA1: 37676322 bp. Our PCR-RFLP results verified that there was a significant association between this locus and the height at the withers in this second sample set (p-value < 0.0005). Horses carrying the GG or AG genotypes at *ANKRD1* were taller on average (mean = 34.36, SD= 2.31) than individuals with the AA genotype (mean= 32.76, SD=2.31) (**Figure 3.5**).

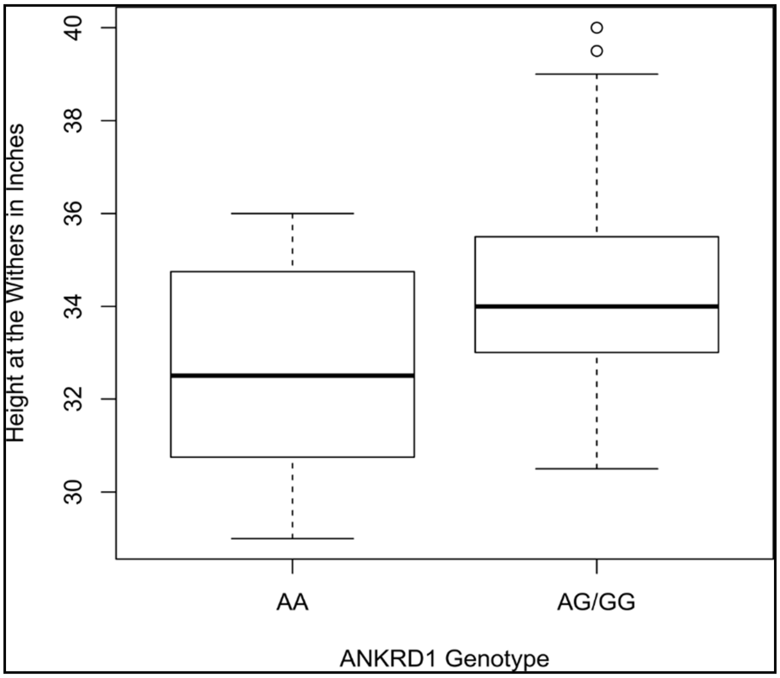


Figure 3.5. Boxplot at *ANKRD1* locus (at ECA1: 37676322 bp) genotypes by withers height (in inches) using a dominant model. Horses with the GG or GA genotypes are on average 1.6 inches (4.064 cm) taller than those possessing the AA genotype horses.

CONCLUSIONS

In the present study we have identified QTLs contributing to size variation in the horse. Some of the significant QTL markers discovered were located within or near genes previously reported to influence size variation in the horse, while others are reported for the first time. Among the newly discovered QTLs affecting the withers height phenotype, the A>G SNP in an intron of the *ANKRD1* gene at ECA1: 37676322 bp variant was verified in an independent set of 90 American Miniature horses. The *ANKRD1* gene is a transcriptional factor that is apparently involved in determining the overall size by affecting muscle growth and differentiation. Further investigation is required to determine the mechanism by which *ANKRD1* affects the overall size in the horse.

ACKNOWLEDGEMENTS

We would like to thank the many horse owners for volunteering to provide samples for DNA and measurements of their horses. We would also like to thank Allison Seebald for assistance in processing the DNA samples. This work was supported by the USDA National Institute of Food and Agriculture, Hatch project NYC-127454. Any opinions, findings, conclusions, or recommendations expressed in this publication are those of the author(s) and do not necessarily reflect the view of the National Institute of Food and Agriculture (NIFA) or the United States Department of Agriculture (USDA).

REFERENCES

Aihara Y., Kurabayashi M., Saito Y., Ohyama Y., Tanaka T., Takeda S., Tomaru K., Sekiguchi K., Arai M., Nakamura T. & Nagai R. (2000) Cardiac ankyrin repeat protein is a novel marker of cardiac hypertrophy: role of M-CAT element within the promoter. *Hypertension* **36**, 48-53.

American Miniature Horse Association (Accessed April 28, 2014) American Miniature Horse Association Rule Book. In: http://www.amha.org/.

Andersson L., From the Science for Life Laboratory D.o.M.B.a.M., Uppsala University, and Department of Animal Breeding and Genetics S.U.o.A.S. & Uppsala S. (2015) How selective sweeps in domestic animals provide new insight into biological mechanisms. *Journal of Internal Medicine* **271**, 1-14.

Arimura T., Bos J.M., Sato A., Kubo T., Okamoto H., Nishi H., Harada H., Koga Y., Moulik M., Doi Y.L., Towbin J.A., Ackerman M.J. & Kimura A. (2009) Cardiac ankyrin repeat protein gene (ANKRD1) mutations in hypertrophic cardiomyopathy. *J Am Coll Cardiol* **54**, 334-42.

Bang M.-L., Gu Y., Dalton N.D., Peterson K.L., Chien K.R. & Chen J. (2014) The Muscle Ankyrin Repeat Proteins CARP, Ankrd2, and DARP Are Not Essential for Normal Cardiac Development and Function at Basal Conditions and in Response to Pressure Overload. *PLoS ONE* **9**, e93638.

Barash I.A., Bang M.L., Mathew L., Greaser M.L., Chen J. & Lieber R.L. (2007) Structural and regulatory roles of muscle ankyrin repeat protein family in skeletal muscle. *Am J Physiol Cell Physiol* **293**, C218-27.

Baumeister A., Arber S. & Caroni P. (1997) Accumulation of muscle ankyrin repeat protein transcript reveals local activation of primary myotube endcompartments during muscle morphogenesis. Journal of Cell Biology **139**, 1231-42.

Bell J.L., Wächter K., Mühleck B., Pazaitis N., Köhn M., Lederer M. & Hüttelmaier S. (2013) Insulin-like growth factor 2 mRNA-binding proteins (IGF2BPs): post-transcriptional drivers of cancer progression? *Cell Mol Life Sci* **70**, 2657-75.

Boyko A.R., Quignon P., Li L., Schoenebeck J.J., Degenhardt J.D., Lohmueller K.E., Zhao K.Y., Brisbin A., Parker H.G., vonHoldt B.M., Cargill M., Auton A., Reynolds A., Elkahloun A.G., Castelhano M., Mosher D.S., Sutter N.B., Johnson G.S., Novembre J., Hubisz M.J., Siepel A., Wayne R.K., Bustamante C.D. & Ostrander E.A. (2010) A Simple Genetic Architecture Underlies Morphological Variation in Dogs. PLoS biology **8**.

Brants J.R., Ayoubi T.A.Y., Chada K., Marchal K., Van de Ven W.J.M. & Petit M.M.R. (2004) Differential regulation of the insulin-like growth factor II mRNA-binding protein genes by architectural transcription factor HMGA2. FEBS letters **569**, 277-83.

Brooks S.A., Gabreski N., Miller D., Brisbin A., Brown H.E., Streeter C., Mezey J., Cook D. & Antczak D.F. (2010a) Whole-genome SNP association in the horse: identification of a deletion in myosin Va responsible for Lavender Foal Syndrome. *PLoS genetics* **6**, e1000909-e.

Brooks S.a., Makvandi-Nejad S., Chu E., Allen J.J., Streeter C., Gu E., McCleery B., Murphy B.a., Bellone R. & Sutter N.B. (2010b) Morphological variation in the horse: defining complex traits of body size and shape. *Animal Genetics* **41 Suppl 2**, 159-65.

Chen H., Patterson N. & Reich D. (2010) Population differentiation as a test for selective sweeps. *Genome Res* **20**, 393-402.

Chen H.J., Lin C.M., Lin C.S., Perez-Olle R., Leung C.L. & Liem R.K. (2006) The role of microtubule actin cross-linking factor 1 (MACF1) in the Wnt signaling pathway. *Genes Dev* **20**, 1933-45.

Christiansen J., Kolte A.M., Hansen T.V.O. & Nielsen F.C. (2009) IGF2 mRNA-binding protein 2: biological function and putative role in type 2 diabetes. *Journal of Molecular Endocrinology* **43**, 187-95.

Cook D., Gallagher P.C. & Bailey E. (2010) Genetics of swayback in American Saddlebred horses. *Animal Genetics* **41 Suppl 2**, 64-71.

Duboscq-Bidot L., Charron P., Ruppert V., Fauchier L., Richter A., Tavazzi L., Arbustini E., Wichter T., Maisch B., Komajda M., Isnard R. & Villard E. (2009) Mutations in the ANKRD1 gene encoding CARP are responsible for human dilated cardiomyopathy. In: *Eur Heart J* (pp. 2128-36, England.

Fedele M., Battista S., Kenyon L., Baldassarre G., Fidanza V., Klein-Szanto A.J., Parlow A.F., Visone R., Pierantoni G.M., Outwater E., Santoro M., Croce C.M. & Fusco A. (2002) Overexpression of the HMGA2 gene in transgenic mice leads to the onset of pituitary adenomas. *Oncogene* **21**, 3190-8.

Friday B.B., Horsley V. & Pavlath G.K. (2000) Calcineurin activity is required for the initiation of skeletal muscle differentiation. *J Cell Biol* **149**, 657-66.

Gianola D., Simianer H. & Qanbari S. (2010) A two-step method for detecting selection signatures using genetic markers. *Genet Res (Camb)* **92**, 141-55.

Groenewoud M.J., Dekker J.M., Fritsche A., Reiling E., Nijpels G., Heine R.J., Maassen J.A., Machicao F., Schafer S.A., Haring H.U., t Hart L.M. & van Haeften T.W. (2008) Variants of CDKAL1 and IGF2BP2 affect first-phase insulin secretion during hyperglycaemic clamps. *Diabetologia* **51**, 1659-63.

Guo J., Jorjani H. & Carlborg Ö. (2012) A genome-wide association study using international breeding-evaluation data identifies major loci affecting production traits and stature in the Brown Swiss cattle breed. *BMC genetics* **13**, 82.

Hansen T.V.O., Hammer N.A., Nielsen J., Madsen M., Dalbaeck C., Wewer U.M., Christiansen J. & Nielsen F.C. (2004) Dwarfism and impaired gut development in insulin-like growth factor II mRNA-binding protein 1-deficient mice. *Molecular and Cellular Biology* **24**, 4448-64.

Jarvis J.P., Scheinfeldt L.B., Soi S., Lambert C., Omberg L., Ferwerda B., Froment A., Bodo J.-M., Beggs W., Hoffman G., Mezey J. & Tishkoff S.A. (2012) Patterns of Ancestry, Signatures of Natural Selection, and Genetic Association with Stature in Western African Pygmies. *PLoS Genet* **8**, e1002641.

Kang H.M., Zaitlen N.a., Wade C.M., Kirby A., Heckerman D., Daly M.J. & Eskin E. (2008) Efficient control of population structure in model organism association mapping. *Genetics* **178**, 1709-23.

Kojic S., Radojkovic D. & Faulkner G. (2011) Muscle ankyrin repeat proteins: their role in striated muscle function in health and disease. *Crit Rev Clin Lab Sci* **48**, 269-94.

Li Z., Gilbert Jason A., Zhang Y., Zhang M., Qiu Q., Ramanujan K., Shavlakadze T., Eash John K., Scaramozza A., Goddeeris Matthew M., Kirsch David G., Campbell Kevin P., Brack Andrew S. & Glass David J. (2012) An HMGA2-IGF2BP2 Axis Regulates Myoblast Proliferation and Myogenesis. *Developmental Cell* **23**, 1176-88.

Ligon A.H., Moore S.D., Parisi M.A., Mealiffe M.E., Harris D.J., Ferguson H.L., Quade B.J. & Morton C.C. (2005) Constitutional rearrangement of the architectural factor HMGA2: a novel human phenotype including overgrowth and lipomas. *Am J Hum Genet* **76**, 340-8.

Ma Y., Zhang H., Zhang Q. & Ding X. (2014) Identification of Selection Footprints on the X Chromosome in Pig. *PLoS ONE* **9**, e94911.

Makvandi-Nejad S., Hoffman G.E., Allen J.J., Chu E., Gu E., Chandler A.M., Loredo A.I., Bellone R.R., Mezey J.G., Brooks S.a. & Sutter N.B. (2012) Four loci explain 83% of size variation in the horse. *PLoS ONE* **7**, e39929-e.

McRae K.M., McEwan J.C., Dodds K.G. & Gemmell N.J. (2014) Signatures of selection in sheep bred for resistance or susceptibility to gastrointestinal nematodes. *BMC Genomics* **15**, 637.

Merk M., Zierow S., Leng L., Das R., Du X., Schulte W., Fan J., Lue H.Q., Chen Y.B., Xiong H.B., Chagnon F., Bernhagen J., Lolis E., Mor G., Lesur O. & Bucala R. (2011) The D-dopachrome tautomerase (DDT) gene product is a cytokine and functional homolog of macrophage migration inhibitory factor (MIF). *Proceedings of the National Academy of Sciences of the United States of America* **108**, E577-E85.

Nielsen R., Williamson S., Kim Y., Hubisz M.J., Clark A.G. & Bustamante C. (2005) Genomic scans for selective sweeps using SNP data. *Genome research* **15**, 1566-75.

Paradis E. (2010) pegas: an R package for population genetics with an integrated-modular approach. *Bioinformatics (Oxford, England)* **26**, 419-20.

Pérez O’Brien A.M., Utsunomiya Y.T., Mészáros G., Bickhart D.M., Liu G.E., Van Tassell C.P., Sonstegard T.S., Da Silva M.V., Garcia J.F. & Sölkner J. (2014) Assessing signatures of selection through variation in linkage disequilibrium between taurine and indicine cattle. *Genetics Selection Evolution* **46**, 19.

Petersen J.L., Mickelson J.R., Rendahl A.K., Valberg S.J., Andersson L.S., Axelsson J., Bailey E., Bannasch D., Binns M.M., Borges A.S., Brama P., da Câmara Machado A., Capomaccio S., Cappelli K., Cothran E.G., Distl O., Fox-Clipsham L., Graves K.T., Guérin G., Haase B., Hasegawa T., Hemmann K., Hill E.W., Leeb T., Lindgren G., Lohi H., Lopes M.S., McGivney B.a., Mikko S., Orr N., Penedo M.C.T., Piercy R.J., Raekallio

M., Rieder S., Røed K.H., Swinburne J., Tozaki T., Vaudin M., Wade C.M. & McCue M.E. (2013) Genome-wide analysis reveals selection for important traits in domestic horse breeds. *PLoS genetics* **9**, e1003211-e.

Purcell S., Neale B., Todd-Brown K., Thomas L., Ferreira Manuel A R., Bender D., Maller J., Sklar P., de Bakker Paul I W., Daly Mark J. & Sham Pak C. (2007) PLINK: A Tool Set for Whole-Genome Association and Population-Based Linkage Analyses. *American journal of human genetics* **81**, 559-75.

Riebler A., Held L. & Stephan W. (2008) Bayesian variable selection for detecting adaptive genomic differences among populations. *Genetics* **178**, 1817-29.

Rubin C.J., Megens H.J., Barrio A.M., Maqbool K., Sayyab S., Schwochow D., Wang C., Carlborg O., Jern P., Jorgensen C.B., Archibald A.L., Fredholm M., Groenen M.A.M. & Andersson L. (2012) Strong signatures of selection in the domestic pig genome. *Proceedings of the National Academy of Sciences of the United States of America* **109**, 19529-36.

Ruyter-Spira C.P., de Groof A.J., van der Poel J.J., Herbergs J., Masabanda J., Fries R. & Groenen M.A. (1998) The HMGI-C gene is a likely candidate for the autosomal dwarf locus in the chicken. *J Hered* **89**, 295-300.

Saastamoinen M. (1990) Heritabilities for Body Size and Growth-Rate and Phenotypic Correlations among Measurements in Young Horses. *Acta Agriculturae Scandinavica* **40**, 377-86.

Sabeti P.C., Varilly P., Fry B., Lohmueller J., Hostetter E., Cotsapas C., Xie X., Byrne E.H., McCarroll S.A., Gaudet R., Schaffner S.F., Lander E.S., Frazer K.A., Ballinger D.G., Cox D.R., Hinds D.A., Stuve L.L., Gibbs R.A., Belmont J.W., Boudreau A., Hardenbol P., Leal S.M., Pasternak S., Wheeler D.A., Willis T.D., Yu F., Yang H., Zeng C., Gao Y., Hu H., Hu W., Li C., Lin W., Liu S., Pan H., Tang X., Wang J., Wang W., Yu J., Zhang B., Zhang Q., Zhao H., Zhao H., Zhou J., Gabriel S.B., Barry R., Blumenstiel B., Camargo A., Defelice M., Faggart M., Goyette M., Gupta S., Moore J., Nguyen H., Onofrio R.C., Parkin M., Roy J., Stahl E., Winchester E., Ziaugra L., Altshuler D., Shen Y., Yao Z., Huang W., Chu X., He Y., Jin L., Liu Y., Shen Y., Sun W., Wang H., Wang Y., Wang Y., Xiong X., Xu L., Waye M.M.Y., Tsui S.K.W., Xue H., Wong J.T.-F., Galver L.M., Fan J.-B., Gunderson K., Murray S.S., Oliphant A.R., Chee M.S., Montpetit A., Chagnon F., Ferretti V., Leboeuf M., Olivier J.-F., Phillips M.S., Roumy S., Sallée C., Verner A., Hudson T.J., Kwok P.-Y., Cai D., Koboldt D.C., Miller R.D., Pawlikowska L., Taillon-Miller P., Xiao M., Tsui L.-C., Mak W., Song Y.Q., Tam P.K.H., Nakamura Y., Kawaguchi T., Kitamoto T., Morizono T., Nagashima A., Ohnishi Y., Sekine A., Tanaka T., Tsunoda T., Deloukas P., Bird C.P., Delgado M., Dermitzakis E.T., Gwilliam R., Hunt S., Morrison J., Powell D., Stranger B.E., Whittaker P., Bentley D.R., Daly M.J., Bakker P.I.W.d., Barrett J., Chretien Y.R., Maller J., McCarroll S., Patterson N., Pe’er I., Price A., Purcell S., Richter D.J., Sabeti P., Saxena R., Sham P.C., Stein L.D., Krishnan L., Smith A.V., Tello-Ruiz M.K., Thorisson G.A., Chakravarti A., Chen P.E., Cutler D.J., Kashuk C.S., Lin S., Abecasis G.R., Guan W., Li Y., Munro H.M., Qin Z.S., Thomas D.J., McVean G., Auton A., Bottolo L., Cardin N., Eyheramendy S., Freeman C., Marchini J., Myers S., Spencer C., Stephens M., Donnelly P., Cardon L.R., Clarke G., Evans D.M., Morris A.P., Weir B.S., Johnson T.A., Mullikin J.C., Sherry S.T., Feolo M., Skol A., Zhang H., Matsuda I., Fukushima Y., Macer D.R., Suda E., Rotimi C.N., Adebamowo C.A., Ajayi I., Aniagwu T., Marshall P.A., Nkwodimmah C., Royal C.D.M., Leppert M.F., Dixon M., Peiffer A., Qiu R., Kent A., Kato K., Niikawa N., Adewole I.F., Knoppers B.M., Foster M.W., Clayton E.W., Watkin J., Muzny D., Nazareth L., Sodergren E., Weinstock G.M., Yakub I., Birren B.W., Wilson R.K., Fulton L.L., Rogers J., Burton J., Carter N.P., Clee C.M., Griffiths M., Jones M.C., McLay K., Plumb R.W., Ross M.T., Sims S.K., Willey D.L., Chen Z., Han H., Kang L., Godbout M., Wallenburg J.C., L'Archevêque P., Bellemare G., Saeki K., Wang H., An D., Fu H., Li Q., Wang Z., Wang R., Holden A.L., Brooks L.D., McEwen J.E., Guyer M.S., Wang V.O., Peterson J.L., Shi M., Spiegel J., Sung L.M., Zacharia L.F., Collins F.S., Kennedy K., Jamieson R. & Stewart J. (2007) Genome-wide detection and characterization of positive selection in human populations. *Nature* **449**, 913-8.

Samaras S.E., Almodovar-Garcia K., Wu N., Yu F. & Davidson J.M. (2015) Global deletion of Ankrd1 results in a wound-healing phenotype associated with dermal fibroblast dysfunction. *Am J Pathol* **185**, 96-109.

Saxena R., Voight B.F., Lyssenko V., Burtt N.P., de Bakker P.I.W., Chen H., Roix J.J., Kathiresan S., Hirschhorn J.N., Daly M.J., Hughes T.E., Groop L., Altshuler D., Almgren P., Florez J.C., Meyer J., Ardlie K., Bostrom K.B., Isomaa B., Lettre G., Lindblad U., Lyon H.N., Melander O., Newton-Cheh C., Nilsson P., Orho-Melander M., Rastam L., Speliotes E.K., Taskinen M.R., Tuomi T., Guiducci C., Berglund A., Carlson J., Gianniny L., Hackett R., Hall L., Holmkvist J., Laurila E., Sjogren M., Sterner M., Surti A., Svensson M., Svensson M., Tewhey R., Blumenstiel B., Parkin M., DeFelice M., Barry R., Brodeur W., Camarata J., Chia N., Fava M., Gibbons J., Handsaker B., Healy C., Nguyen K., Gates C., Sougnez C., Gage D., Nizzari M., Gabriel S.B., Chirn G.W., Ma Q.C., Parikh H., Richardson D., Ricke D., Purcell S., In D.G.I.B. & Res N.I.B. (2007) Genome-wide association analysis identifies loci for type 2 diabetes and triglyceride levels. *Science* **316**, 1331-6.

Shin J. & Lee C. (2015) A mixed model reduces spurious genetic associations produced by population stratification in genome-wide association studies. *Genomics* **105**, 191-6.

Signer-Hasler H., Flury C., Haase B., Burger D., Simianer H., Leeb T. & Rieder S. (2012) A Genome-Wide Association Study Reveals Loci Influencing Height and Other Conformation Traits in Horses. *PLoS ONE* **7**, e37282.

Sutter N.B., Bustamante C.D., Chase K., Gray M.M., Zhao K., Zhu L., Padhukasahasram B., Karlins E., Davis S., Jones P.G., Quignon P., Johnson G.S., Parker H.G., Fretwell N., Mosher D.S., Lawler D.F., Satyaraj E., Nordborg M., Lark K.G., Wayne R.K. & Ostrander E.A. (2007) A Single IGF1 Allele Is a Major Determinant of Small Size in Dogs. *Science* **316**, 112-5.

Tajima F. (1989) Statistical-Method for Testing the Neutral Mutation Hypothesis by DNA Polymorphism. *Genetics* **123**, 585-95.

Tu J., Chen Y.L., Cai L.L., Xu C.M., Zhang Y., Chen Y.M., Zhang C., Zhao J., Cheng J.K., Xie H.W., Zhong F. & He F.C. (2015) Functional Proteomics Study Reveals SUMOylation of TFII-I is Involved in Liver Cancer Cell Proliferation. *Journal of Proteome Research* **14**, 2385-97.

Voight B.F., Kudaravalli S., Wen X. & Pritchard J.K. (2006) A Map of Recent Positive Selection in the Human Genome. *PLoS Biol* **4**, e72.

Wade C.M., Giulotto E., Sigurdsson S., Zoli M., Gnerre S., Imsland F., Lear T.L., Adelson D.L., Bailey E., Bellone R.R., Blöcker H., Distl O., Edgar R.C., Garber M., Leeb T., Mauceli E., MacLeod J.N., Penedo M.C.T., Raison J.M., Sharpe T., Vogel J., Andersson L., Antczak D.F., Biagi T., Binns M.M., Chowdhary B.P., Coleman S.J., Della Valle G., Fryc S., Guérin G., Hasegawa T., Hill E.W., Jurka J., Kiialainen a., Lindgren G., Liu J., Magnani E., Mickelson J.R., Murray J., Nergadze S.G., Onofrio R., Pedroni S., Piras M.F., Raudsepp T., Rocchi M., Røed K.H., Ryder O.a., Searle S., Skow L., Swinburne J.E., Syvänen a.C., Tozaki T., Valberg S.J., Vaudin M., White J.R., Zody M.C., Lander E.S. & Lindblad-Toh K. (2009) Genome sequence, comparative analysis, and population genetics of the domestic horse. *Science (New York, N.Y.)* **326**, 865-7.

Weedon M.N., Lettre G., Freathy R.M., Lindgren C.M., Voight B.F., Perry J.R.B., Elliott K.S., Hackett R., Guiducci C., Shields B., Zeggini E., Lango H., Lyssenko V., Timpson N.J., Burtt N.P., Rayner N.W., Saxena R., Ardlie K., Tobias J.H., Ness A.R., Ring S.M., Palmer C.N.A., Morris A.D., Peltonen L., Salomaa V., Smith G.D., Groop L.C., Hattersley A.T., McCarthy M.I., Hirschhorn J.N. & Frayling T.M. (2007) A common variant of HMGA2 is associated with adult and childhood height in the general population. *Nature genetics* **39**, 1245-50.

Weir B.S. & Cockerham C.C. (1984) Estimating F-Statistics for the Analysis of Population Structure. *Evolution* **38**, 1358-70.

Wood A.R., Esko T., Yang J., Vedantam S., Pers T.H., Gustafsson S., Chu A.Y., Estrada K., Luan J.a., Kutalik Z., Amin N., Buchkovich M.L., Croteau-Chonka D.C., Day F.R., Duan Y., Fall T., Fehrmann R., Ferreira T., Jackson A.U., Karjalainen J., Lo K.S., Locke A.E., Mägi R., Mihailov E., Porcu E., Randall J.C., Scherag A., Vinkhuyzen A.A.E., Westra H.-J., Winkler T.W., Workalemahu T., Zhao J.H., Absher D., Albrecht E., Anderson D., Baron J., Beekman M., Demirkan A., Ehret G.B., Feenstra B., Feitosa M.F., Fischer K., Fraser R.M., Goel A., Gong J., Justice A.E., Kanoni S., Kleber M.E., Kristiansson K., Lim U., Lotay V., Lui J.C., Mangino M., Leach I.M., Medina-Gomez C., Nalls M.A., Nyholt D.R., Palmer C.D., Pasko D., Pechlivanis S., Prokopenko I., Ried J.S., Ripke S., Shungin D., Stancáková A., Strawbridge R.J., Sung Y.J., Tanaka T., Teumer A., Trompet S., Laan S.W.v.d., Setten J.v., Vliet-Ostaptchouk J.V.V., Wang Z., Yengo L., Zhang W., Afzal U., Ärnlöv J., Arscott G.M., Bandinelli S., Barrett A., Bellis C., Bennett A.J., Berne C., Blüher M., Bolton J.L., Böttcher Y., Boyd H.A., Bruinenberg M., Buckley B.M., Buyske S., Caspersen I.H., Chines P.S., Clarke R., Claudi-Boehm S., Cooper M., Daw E.W., Jong P.A.D., Deelen J., Delgado G., Denny J.C., Dhonukshe-Rutten R., Dimitriou M., Doney A.S.F., Dörr M., Eklund N., Eury E., Folkersen L., Garcia M.E., Geller F., Giedraitis V., Go A.S., Grallert H., Grammer T.B., Gräßler J., Grönberg H., Groot L.C.P.G.M.d., Groves C.J., Haessler J., Hall P., Haller T., Hallmans G., Hannemann A., Hartman C.A., Hassinen M., Hayward C., Heard-Costa N.L., Helmer Q., Hemani G., Henders A.K., Hillege H.L., Hlatky M.A., Hoffmann W., Hoffmann P., Holmen O., Houwing-Duistermaat J.J., Illig T., Isaacs A., James A.L., Jeff J., Johansen B., Johansson Å., Jolley J., Juliusdottir T., Junttila J., Kho A.N., Kinnunen L., Klopp N., Kocher T., Kratzer W., Lichtner P., Lind L., Lindström J., Lobbens S., Lorentzon M., Lu Y., Lyssenko V., Magnusson P.K.E., Mahajan A., Maillard M., McArdle W.L., McKenzie C.A., McLachlan S., McLaren P.J., Menni C., Merger S., Milani L., Moayyeri A., Monda K.L., Morken M.A., Müller G., Müller-Nurasyid M., Musk A.W., Narisu N., Nauck M., Nolte I.M., Nöthen M.M., Oozageer L., Pilz S., Rayner N.W., Renstrom F., Robertson N.R., Rose L.M., Roussel R., Sanna S., Scharnagl H., Scholtens S., Schumacher F.R., Schunkert H., Scott R.A., Sehmi J., Seufferlein T., Shi J., Silventoinen K., Smit J.H., Smith A.V., Smolonska J., Stanton A.V., Stirrups K., Stott D.J., Stringham H.M., Sundström J., Swertz M.A., Syvänen A.-C., Tayo B.O., Thorleifsson G., Tyrer J.P., Dijk S.v., Schoor N.M.v., Velde N.v.d., Heemst D.v., Oort F.V.A.v., Vermeulen S.H., Verweij N., Vonk J.M., Waite L.L., Waldenberger M., Wennauer R., Wilkens L.R., Willenborg C., Wilsgaard T., Wojczynski M.K., Wong A., Wright A.F., Zhang Q., Arveiler D., Bakker S.J.L., Beilby J., Bergman R.N., Bergmann S., Biffar R., Blangero J., Boomsma D.I., Bornstein S.R., Bovet P., Brambilla P., Brown M.J., Campbell H., Caulfield M.J., Chakravarti A., Collins R., Collins F.S., Crawford D.C., Cupples L.A., Danesh J., Faire U.d., Ruijter H.M.d., Erbel R., Erdmann J., Eriksson J.G., Farrall M., Ferrannini E., Ferrières J., Ford I., Forouhi N.G., Forrester T., Gansevoort R.T., Gejman P.V., Gieger C., Golay A., Gottesman O., Gudnason V., Gyllensten U., Haas D.W., Hall A.S., Harris T.B., Hattersley A.T., Heath A.C., Hengstenberg C., Hicks A.A., Hindorff L.A., Hingorani A.D., Hofman A., Hovingh G.K., Humphries S.E., Hunt S.C., Hypponen E., Jacobs K.B., Jarvelin M.-R., Jousilahti P., Jula A.M., Kaprio J., Kastelein J.J.P., Kayser M., Kee F., Keinanen-Kiukaanniemi S.M., Kiemeney L.A., Kooner J.S., Kooperberg C., Koskinen S., Kovacs P., Kraja A.T., Kumari M., Kuusisto J., Lakka T.A., Langenberg C., Marchand L.L., Lehtimäki T., Lupoli S., Madden P.A.F., Männistö S., Manunta P., Marette A., Matise T.C., McKnight B., Meitinger T., Moll F.L., Montgomery G.W., Morris A.D., Morris A.P., Murray J.C., Nelis M., Ohlsson C., Oldehinkel A.J., Ong K.K., Ouwehand W.H., Pasterkamp G., Peters A., Pramstaller P.P., Price J.F., Qi L., Raitakari O.T., Rankinen T., Rao D.C., Rice T.K., Ritchie M., Rudan I., Salomaa V., Samani N.J., Saramies J., Sarzynski M.A., Schwarz P.E.H., Sebert S., Sever P., Shuldiner A.R., Sinisalo J., Steinthorsdottir V., Stolk R.P., Tardif J.-C., Tönjes A., Tremblay A., Tremoli E., Virtamo J., Vohl M.-C., Consortium T.E.M.R.a.G.e., Consortium T.M., Consortium T.P., Study T.L.C., Amouyel P., Asselbergs F.W., Assimes T.L., Bochud M., Boehm B.O., Boerwinkle E., Bottinger E.P., Bouchard C., Cauchi S., Chambers J.C., Chanock S.J., Cooper R.S., Bakker P.I.W.d., Dedoussis G., Ferrucci L., Franks P.W., Froguel P., Groop L.C., Haiman C.A., Hamsten A., Hayes M.G., Hui J., Hunter D.J., Hveem K., Jukema J.W., Kaplan R.C., Kivimaki M., Kuh D., Laakso M., Liu Y., Martin N.G., März W., Melbye M., Moebus S., Munroe P.B., Njølstad I., Oostra B.A., Palmer C.N.A., Pedersen N.L., Perola M., Pérusse L., Peters U., Powell J.E., Power C., Quertermous T., Rauramaa R., Reinmaa E., Ridker P.M., Rivadeneira F., Rotter J.I., Saaristo T.E., Saleheen D., Schlessinger D., Slagboom P.E., Snieder H., Spector T.D., Strauch K., Stumvoll M., Tuomilehto J., Uusitupa M., Harst P.v.d., Völzke H., Walker M., Wareham N.J., Watkins H., Wichmann H.-E., Wilson J.F., Zanen P., Deloukas P., Heid I.M., Lindgren C.M., Mohlke K.L., Speliotes E.K., Thorsteinsdottir U., Barroso I., Fox C.S., North K.E., Strachan D.P., Beckmann J.S., Berndt S.I., Boehnke M., Borecki I.B., McCarthy M.I., Metspalu A., Stefansson K., Uitterlinden A.G., Duijn C.M.v., Franke L., Willer C.J., Price A.L., Lettre G., Loos R.J.F., Weedon M.N., Ingelsson E., O'Connell J.R., Abecasis G.R., Chasman D.I., Goddard M.E., Visscher P.M., Hirschhorn J.N. & Frayling T.M. (2014) Defining the role of common variation in the genomic and biological architecture of adult human height. *Nature genetics* **46**, 1173-86.

Wright S. (1949) THE GENETICAL STRUCTURE OF POPULATIONS. *Annals of Eugenics* **15**, 323-54.

Young A.R. & Narita M. (2007) Oncogenic HMGA2: short or small? Genes Dev **21**, 1005-9.

Zhou X.J., Benson K.F., Ashar H.R. & Chada K. (1995) Mutation Responsible for the Mouse Pygmy Phenotype in the Developmentally-Regulated Factor Hmgi-C. *Nature* **376**, 771-4.

CHAPTER 4

ESTIMATION OF GENOME-WIDE INBREEDING AND POPULATION STRUCTURE IN AN ARABIAN HORSE HERD

M.A Al Abri[1, 2], U. König von Borstel[3], V. Strecker[3], S. A. Brooks[1*]

[1]Department of Animal Science, Cornell University, Ithaca, NY 14853, USA

[2]Department of Animal and Veterinary Sciences,College of Agriculture and Marine Sciences,

Sultan Qaboos University, PO box 34 Al Khod, Postal Code 123, Muscat, Oman

[3]Department of Animal Breeding and Genetics, Göttingen University. Göttingen, Germany

*Corresponding author

[*]Now Department of Animal Sciences, University of Florida, Gainesville, FL 32611, USA

ABSTRACT

Horse breeders rely heavily on the accurate identification of individual ancestry through pedigrees. Errors in such pedigrees may inaccurately assign horses to false lineages or breed memberships, and can result in inaccurate estimates of inbreeding. Moreover, discrepancies in pedigree records can lead horse owners into making misguided purchasing and breeding decisions. Genome-wide SNP data provides a robust and quantitative tool to resolve lineage assignments errors and provide genomic measures of inbreeding. The aim of this project was to pilot a comparison between pedigree and genomic relatedness and inbreeding measures in a closed herd. Here, we describe a herd of 36 pedigreed Egyptian Arabian horses genotyped using the Equine SNP70 (Geneseek, Inc.). Genomic inbreeding values and pair-wise relatedness between horses within the herd were estimated from the genotypic data. Multi-dimensional Scaling Analysis (MDS), and clustering analysis were performed to describe the relationships within the herd and the results were compared to the pedigree information. Pedigree inbreeding values had a moderate but significant correlation with the genomic inbreeding values ($r = 0.406$, $p=0.014$). Conversely, the correlation was higher between genomic relationships and pedigree relationships ($r=0.77$, $p<0.005$). Although first degree relationships between individuals were successfully reconstructed, more distant relationships were more difficult to resolve. In comparing the herd to a sample of US, Polish and Egyptian Arabian horse populations the herds' historically recorded Egyptian lineage was successfully recovered among other Arabian horse sub-groups. Our conclusion is that genome-wide genotypes are superior to pedigree derived inbreeding predictions and have utility in verification of the integrity of pedigrees where records are unavailable or in doubt. Although still considered costly, application of genomic tools in a breeding program can be advantageously utilized to measure inbreeding in valuable lines of

horse, and in breeds already at risk for loss of genomic diversity.

INTRODUCTION

Throughout a long history alongside humans Arabian horses were selected for their endurance, intelligence and beauty (Porter V 2002). Therefore, the Arabian horse breed contributed during the development of many of the modern horse breeds like the Thoroughbred (Bower *et al.* 2012) , Marwari (Jun *et al.* 2014), French horses (Pirault *et al.* 2013) and a number of Spanish Arabian horse breeds (Cervantes *et al.* 2009). Yet, in recent history stringent selective breeding within the Arabian horse may result in a loss of heterozygosity within the breed, and potentially inbreeding depression. Arabian horses tend to have relatively high inbreeding levels (Pirault *et al.* 2013) which may be the result of the intentional mating of relatives within the breed (Moureaux *et al.* 1996). In Arabian horses, inbreeding has resulted in elevation of the incidence of recessive genetic disorders such as Lavender Foal Syndrome (Brooks *et al.* 2010a) and Severe Combined Immunodeficiency (Wiler *et al.* 1995). Presently, horse breeders can utilize commercially available single-locus tests for a number of genetic disorders to make informed breeding decisions. However, the need for precise quantification of inbreeding is imperative for maintenance of genetic diversity and avoidance of yet undiscovered recessive conditions. Moreover, high inbreeding levels can result in a reduced fitness of the population as a whole.

Incomplete or erroneous pedigrees may lead to inaccurate estimates of the inbreeding coefficient (Mucha & Windig 2009). Furthermore, the reliability of inbreeding estimates from pedigrees depends largely on its depth (Lutaaya *et al.* 1999). Even in the presence of deep pedigrees, like those available for the Arabian Horse, these estimates could be of a limited value as they converge to one as the pedigree depth increases unless the pedigree is truncated at some generation in the past (Speed & Balding 2014).

In recent years, the availability of equine genome-wide SNP genotyping platforms provided an alternative to estimate inbreeding and relationships measures without relying on pedigrees. This had enabled the comparison of genomic inbreeding levels between horse breeds (McCue *et al.* 2012) and to assess its extent within a given breed (Binns *et al.* 2012). In this project, we compared pedigree inbreeding and relationship values to their genomic counterparts in a herd of 36 Arabian horses genotyped using the Equine SNP70 (Illumina Inc). Additionally, we compared various clustering and genetic similarity measures within the herd and in comparison to Arabian horses from a diverse background.

MATERIALS AND METHODS

Description of the herd

This herd provides an excellent test population for this comparison due to the availability of high quality pedigrees and a recent bottleneck event following closure of the herd to outside bloodstock in 1981. Pedigree information was obtained for the 36 horses of the herd from the German National Genetic Evaluation Centre and traced back to 1840, yielding a total of 1130 ancestors born between 1840 and 2013. A detailed description of the herd's pedigree is given in **Table 4.1**.

Table 4.2. Summary of the pedigree used for the inbreeding and relationships calculation.

Category	**Count**
Number of Sires	374
Number of Dams	534
Number of Sires' progeny	898
Number of Dams' progeny	880
Individuals with no progeny	222
Number of individuals with both known parents	874

Pedigree Completeness DNA extraction and genotyping

36 Arabian horses representing the last three generations in the pedigree were chosen for genotyping (**Figure 4.1**). DNA was extracted from hair following a modified Puregene protocol (Cook *et al.* 2010). The DNA samples were then genotyped using the equine SNP 70 Illumina BeadChip (GeneSeek, Lincoln, NE, USA). Additionally, 36 US Arabian, 15 Egyptian Arabian and 11 Polish Arabian horses that are part of an unpublished study were genotyped using SNP 50 Illumina BeadChip (GeneSeek, Lincoln, NE, USA).

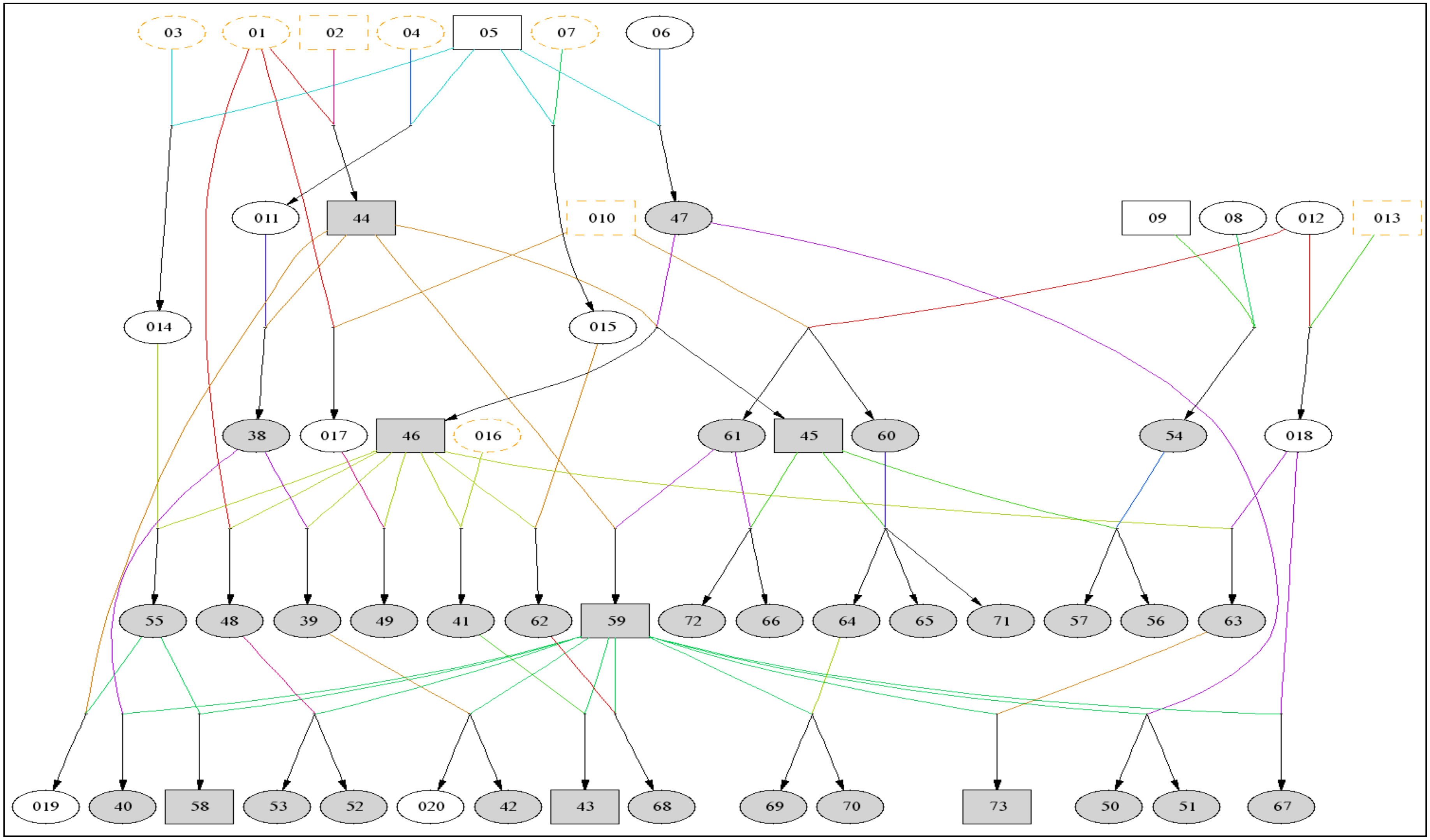


Figure 4.1: Pedigree of sampled population of horses. Grey shading denotes individuals chosen for genotyping; ovals are females and rectangles males.

To ensure a fair comparison between the pedigree and genomic inbreeding values, we sought to assess the extent of the pedigree completeness for all the genotyped individuals. Therefore, the pedigree completeness index (PCI) was calculated using three generations back using the method proposed by MacCluer *et al.* (1983). In order to void animals with less complete pedigrees, we chose the year 1941 as reasonable truncation point in the pedigree and calculated PCI values for animals born in that year onwards.

For each animals, PCI =$\frac{2C_{sire}\, C_{dam}}{C_{sire}+C_{dam}}$

Where the terms C_{sire} and C_{dam} correspond to the contributions from the paternal and maternal lines respectively.

$$C=\frac{1}{g}\sum_{i=1}^{g} a_i$$

a_i is the proportion of known ancestors in generation i; and g is the number of generations considered ($g = 3$).

Pedigree based inbreeding and pair-wise relationships

Pedigree-based inbreeding was calculated using the indirect method proposed by (Colleau 2002) using the program CFC (Sargolzaei *et al.* 2006). Pair-wise pedigree relationships based on the additive numerator relationship matrix were also calculated using CFC. We included animals born in the year 1941 and onwards to avoid over estimating pair-wise relatedness between individuals.

Genotypes summary, filtering and genomic inbreeding calculation

The Equine SNP70 array produced genotypes for 65,157 markers. As a quality control, markers with a minor allele frequency (MAF) less than 10% or with a missingness rate greater than 20% were excluded from the analysis. This reduced the number of markers to 25666 markers. We further pruned the data by linkage disequilibrium before calculating inbreeding values such that we have a set of markers that is in linkage equilibrium. This was done in order to avoid over-estimating inbreeding due to areas in the bead-chip where markers in tight LD occur in higher density. In the pruning process, we randomly discarded one of a pair of markers in a given window of 50 markers (step size 5 markers) if the LD between them was larger than r^2=90. After pruning by LD, 7217 markers remained for the analysis. Genomic inbreeding and homozygosity values for each animal were then calculated based on these markers in PLINK (v1.07) (Purcell *et al.* 2007) using the command --*het*. The percent homozygosity was calculated as the ratio of the observed homozygous markers to the total number of markers.

Estimation of the minimum number of markers sufficient for genomic inbreeding calculation using bootstrap

We investigated the ability of various sizes of reduced sets of randomly chosen SNPs (n=500, 1000, 2000, 3000 and 5000) in obtaining genomic inbreeding estimates comparable to those obtained using the full set of markers in linkage equilibrium. 1000 samples were generated of each set size by sampling from the 7217 SNPs in linkage equilibrium using the --*thin* command in PLINK. The gain in accuracy as the number of SNPs was increased was measured using the correlation between the inbreeding values in each sample of a reduced set and the full set in linkage equilibrium. Additionally, we calculated the mean inbreeding for each sample of the reduced SNP set. Overall means of inbreeding values, their correlations with the full set and corresponding 95% confidence intervals were calculated for the 1000 samples of each set in Microsoft Excel (2007).

Pair-wise IBS, genetic distance and genetic background analysis

Genome wide markers have remarkable power to discern cryptic relationships between individuals and detect pedigree errors. In order to leverage that ability, we used PLINK to generate a pair-wise identity by state (IBS) matrix of individuals using the option *--cluster --matrix*.

The --*genome* command then calculates an IBS distance (Dst) metric which represents the proportion of IBS alleles shared and is defined as follows:

$$Dst = \frac{IBS2 + 0.5\ \times IBS1}{N}$$

Where IBS2 and IBS1 are the number of loci that share 2 or 1 alleles IBS, respectively, and N is the number of loci tested.

We used the IBS distance metric (Dst) to calculate the genetic distance (D) between individuals in pair-wise combinations following Ai *et al.* (2013), where D was defined as 1-Dst.

Additionally, we performed a multidimensional scaling analysis (MDS) to assess clustering within group (i.e the herd itself) and for the herd's founders amongst other Arabian horses using the command *--cluster --mds-plot* in PLINK.

In order to further describe relatedness among these horses, we assessed the genetic admixture within the group and for the group's founders amongst other Arabian horses using the bayesian clustering algorithm STRUCTURE (Pritchard *et al.* 2000) using a burn-in period of 10,000 iterations followed by 20,000 iterations from which estimates were obtained. For the choice of the optimal number of putative genetically-defined populations i.e K, 20 structure runs were conducted for each K value from K=1 to K=8. The best K was determined using the method of (Evanno *et al.* 2005) which is based on the change in the log probability of data between successive K values. The analysis showed that the best K value was 3 for the analysis of the herd amongst American, Polish and Egyptian Arabian horses. The within herd analysis best K value was 5. The results were plotted using CLUMPP (Jakobsson & Rosenberg 2007).

Genomic relationship measures

Although we recognize the potential for a better pair-wise relatedness measure from SNPs, we chose the concordance with the pedigree as a criterion to compare between methods as it was previously used as gold standard in other studies e.g Lopes *et al.* (2013b) and (Santure *et al.* 2010). Also, the Arabian horse is a well-documented breed, and we had a deep pedigree with a high PCI for all genotyped animals. We compared the relationship coefficients obtained from the following four software packages to the pedigree relationships:

1) The R package GenABEL (Aulchenko *et al.* 2007) was used to obtain kinships measures weighed by the frequency of the alleles using the *IBS* function.
2) In the program KING (Manichaikul *et al.* 2010), we used the Robust estimator which assumes all individuals are unrelated. For comparison, we also used the *--homo* option which assumes all samples are from a homogeneous population.
3) We used the R package SNPRelate (Zheng *et al.* 2012), to obtain the method of moments as well as the maximum likelihood estimated of pair-wise relatedness using the *snpgdsIBDMoM* (essentially equal to PLINK's IBD relationship estimate i.e PI HAT) and *snpgdsIBDMLE* functions respectively.
4) Pair-wise kinship measures were obtained in GCTA (Yang *et al.* 2011) using the command *--make-grm*.

RESULTS AND DISCUSSION

Pedigree Completeness Index

The average PCI by year of birth was consistently above 85 % after the close of the herd in 1981 (**Figure 4.2)**. For the genotyped animals, the PCI was 100 %. This indicates that for this population the pedigree is of an excellent quality and can be reliably used to compare the inbreeding values derived from it with the genomic measures of inbreeding.

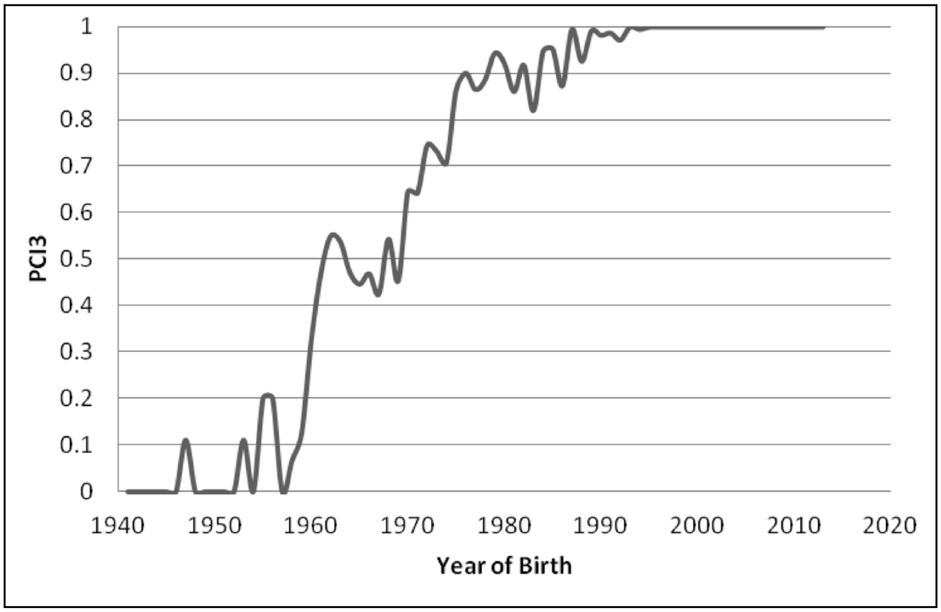


Figure 4.2. Average Pedigree Completeness Index by Year of Birth

Pedigree Inbreeding, Genomic Inbreeding and Homozygosity

There was a significant moderate correlation between pedigree and genomic inbreeding values (r = 0.41, p=0.014) (**Figure 4.3-A**). Yet, the magnitude of this correlation indicates that pedigree inbreeding and genomic inbreeding do not strongly agree with one another. Some individuals with low genomic inbreeding values had relatively high pedigree inbreeding values. Also, a number of individuals with similar pedigree inbreeding values (e.g halfsibs) had very different genomic pedigree values. Such discrepancies can result from errors in the pedigree itself, but are frequently attributed to Mendelian sampling which is ignored in pedigree inbreeding. Also, the small herd size of 36 horses could have contributed to a bias in the estimation of the actual allelic frequencies as these are derived only from the genotypes of these 36 animals. In an ideal situation allelic frequencies in the base population would be used but in practice this is not often possible (Speed & Balding 2014). In a simulation study, use of allele frequencies estimated in the base population resulted in improved agreement between pedigree and genomic estimated inbreeding values (VanRaden 2008). In fact, adjusting for allele frequencies in the base population also made genomic estimated breeding values (GEBVs) more accurate (VanRaden *et al.* 2011). Nevertheless, we used the correlation between pedigree and genomic inbreeding values as an easy way to check the concordance of these two approaches.

The pedigree estimated inbreeding values (F_{PED}), their corresponding genomic inbreeding values (F_{SNP}) and percentage of homozygous markers for each individual are shown in **Table 4.2**. The overwhelming majority of the animals had negative genomic inbreeding values with a mean of -0.184 (SD=0.128). This is a result of the usage of the current population as the base population to estimate allelic frequencies as mentioned above (Powell *et al.* 2010). However, it is indicative that for most animals, the observed homozygosity at the individual level is lower than the expected by chance (Purcell *et al.* (2007) and Powell *et al.* (2010)). Therefore, the inbreeding value of each animal should be interpreted in the context of the rest of the animals in this herd. The genomic inbreeding value and percentage of homozygous markers were very highly correlated (r=0.99, p < 2.2e-16). Indeed the genomic inbreeding estimates implemented in the PLINK software rely on the number of homozygous SNPs expected by chance and the number of homozygous SNPs observed (Purcell *et al.* 2007) .

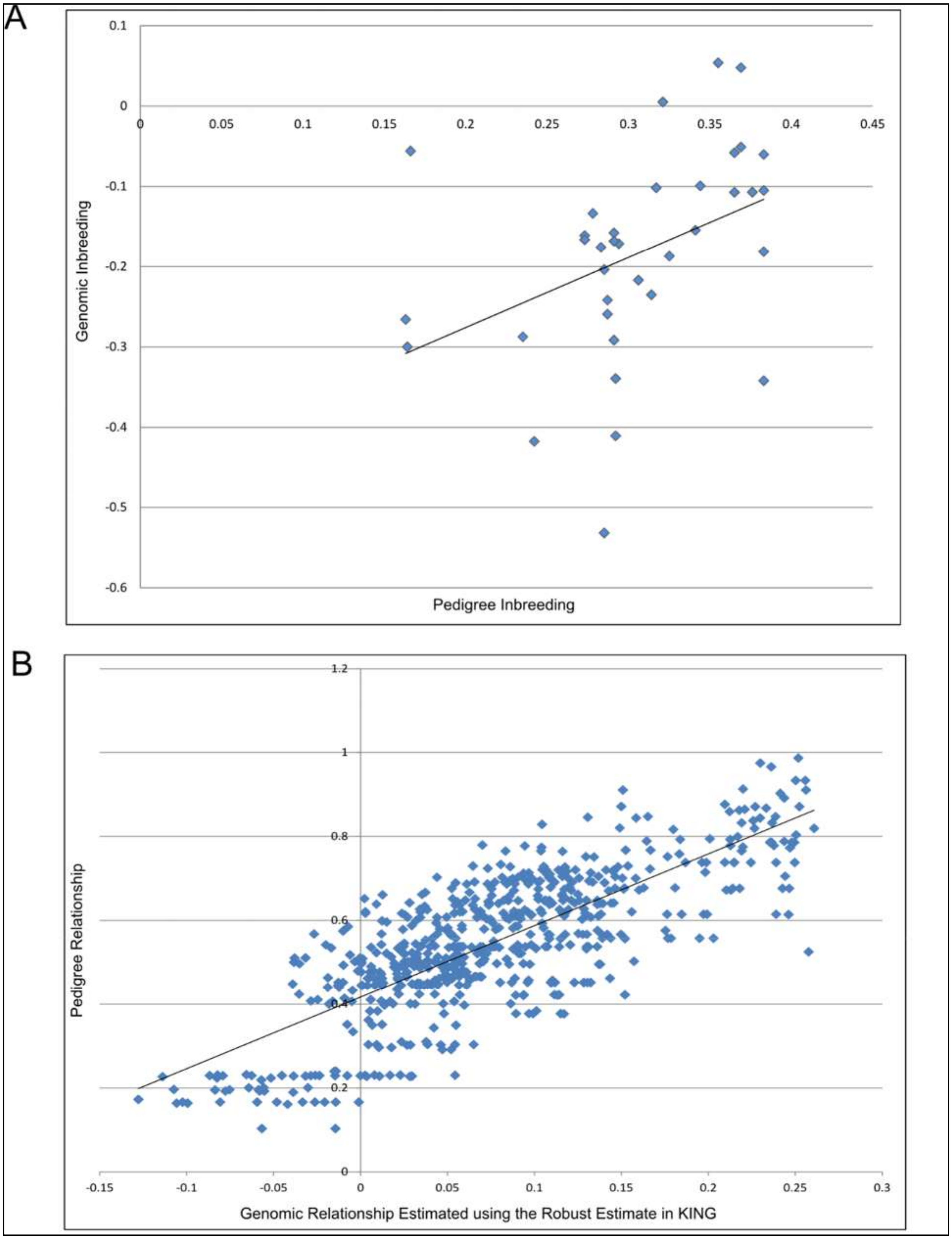


Figure 4.3. **A**. Pedigree Inbreeding vs Genomic Inbreeding values.
B. Pair-wise Pedigree Relationships vs Genomic Relationships.

Table 4.2. Pedigree estimated inbreeding values and percentage of homozygous markers across the genome for each animal in the herd.

Animal ID	Year of Birth	Pedigree inbreeding (F)	Genomic inbreeding	% Homozygosity
42	2013	0.383	-0.342	45.75
44	1996	0.242	-0.4174	42.79
47	1992	0.235	-0.2874	48.26
56	2010	0.163	-0.2658	49.04
46	2005	0.285	-0.5317	38.16
65	2009	0.291	-0.2915	48.02
72	2013	0.292	-0.3393	45.92
66	2010	0.291	-0.4106	42.77
54	2001	0.166	-0.05591	57.53
50	2010	0.287	-0.2592	49.28
41	2007	0.314	-0.2351	50.34
63	2008	0.283	-0.1757	52.76
55	2005	0.278	-0.1335	54.45
43	2012	0.341	-0.1545	53.50
45	2001	0.285	-0.2037	51.64
51	2011	0.287	-0.2417	49.87
71	2013	0.291	-0.1578	53.48
60	1998	0.273	-0.161	53.35
59	2005	0.294	-0.1713	52.89
62	2006	0.325	-0.1869	52.23
61	2001	0.273	-0.1663	53.13
38	2001	0.306	-0.2169	51.03
67	2010	0.317	-0.1016	55.76
53	2013	0.365	-0.1072	55.52
64	2008	0.291	-0.168	52.98
48	2004	0.376	-0.107	55.50
39	2008	0.383	-0.1049	55.50
73	2013	0.383	-0.1812	52.50
49	2005	0.344	-0.09916	55.84
52	2012	0.365	-0.05799	57.50
70	2013	0.369	-0.05088	57.79
40	2009	0.383	-0.06017	57.42
69	2012	0.369	0.04782	61.74
58	2012	0.321	0.005146	60.01
68	2012	0.355	0.05384	61.99

Since pedigree-estimated inbreeding values are not equal to the true inbreeding values (VanRaden *et al.* 2011), they do not perfectly reflect of the level of homozygosity in the genome. For example, horse ID: 42 is predicted based on her pedigree to be among some of the most inbred individuals in the sample set (F_{PED}= 0.383), yet with just 45.75 % homozygous markers, she is below the herd mean of 52.33 %. On the other hand, horse ID: 58, has an F_{PED} value of 0.321 and a genomic homozygosity value of 60.0 %, which is notably higher compared to that of horse ID: 42. Indeed, full siblings with same pedigree inbreeding values have different levels of homozygosity. For instance, horses ID: 64, ID: 71 and ID: 65 are full siblings with identical F_{PED} values of 0.291 but their genomic homozygosity varies from 48.02% to 53.47%. Thus, when considering matings for individual animals rather than a population of individuals, genomic values are more accurate in assessing the level of homozygosity across the genome.

In the horse, selection is often applied though mate choice and culling of very specific individuals, rather than simultaneously across a group or herd. Individual horses can be of high monetary value, and therefore the benefits in increased accuracy of inbreeding measures through genomics may be well worth the added expense. For example, while individuals ID:46 and ID:45 both possessed a pedigree estimated inbreeding value of 0.285, individual ID:46 has a 33 % lower genomic inbreeding value and is likely the better choice as a future breeding animal for the goal of maintaining genetic diversity.

Correlation of birth year and pedigree-estimated inbreeding levels suggested a significant trend toward increasing inbreeding in later foal crops (r= 0.402334, p-value 0.01499) (**Figure 4.4**), which was expected given the closure of the herd in 1981. Yet, birth year was not significantly correlated with either genomic homozygosity (r= 0.223, p= 0.189) nor genomic inbreeding (r= 0.229, p= 0.178). Improved accuracy of genome based calculations may have highlighted an underlying effect of simultaneous selection for overall health. The lack of correlation here is perhaps more reflective of the reality within the herd as there is a rigorous effort to select horses based on health and conformation traits. This selection could be enough to favor individuals with higher heterozygosity and choosing them would therefore maintain genomic diversity within the herd.

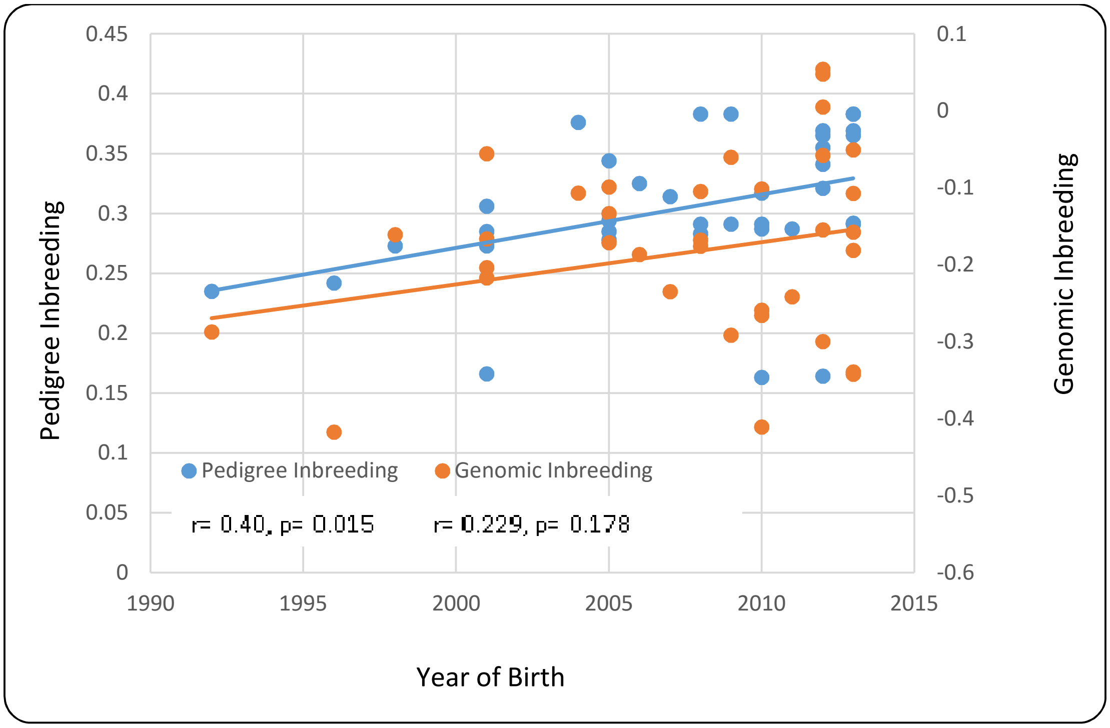


Figure 4.4. Pedigree estimated inbreeding values (F) and genomic inbreeding for the herd over the years.

Estimation of the minimum number of markers sufficient for inbreeding calculation

Although the cost of genotyping by high throughput arrays is falling, application of these technologies in animal production is still economically challenging for most producers. Therefore, determining the minimum number of unlinked loci required to provide a reasonable estimate of inbreeding is important for future development of low-cost genotyping panels. We found that decreasing the number of unlinked SNPs used to measure genomic inbreeding did decrease the correlation of these values with those obtained using the full set of 7219 SNPs in linkage equilibrium (**Figure 4.5**). The correlations ranged from 0.77 to 0.83 for subsets comprised of 500 to 5000 SNPs. The true mean inbreeding value of -0.1843 was captured successfully by all reduced sets, although the 95% confidence interval was 5 times wider in the smallest (500 SNPs, CI = 0.0027) than that in the largest set (5000 SNPs, CI = 0.00052). However, increasing the number of markers from 2000 to 5000 did not considerably increase the correlation, which improved only by 1% (from 0.82 to 0.83). Therefore, we recommend that sets of at least 2000 markers in linkage equilibrium be used for inbreeding estimation in the Arabian horse and breeds of similar structure. The same number of markers was found sufficient for calculating inbreeding in pigs (Lopes *et al.* 2013a) and is very close to the 2500 SNPs suggested for beef cattle to estimate relationships (Rolf *et al.* 2010). However, as other horse breeds and livestock species may possess significantly different haplotype length across the genome, this marker depth is likely not universally appropriate.

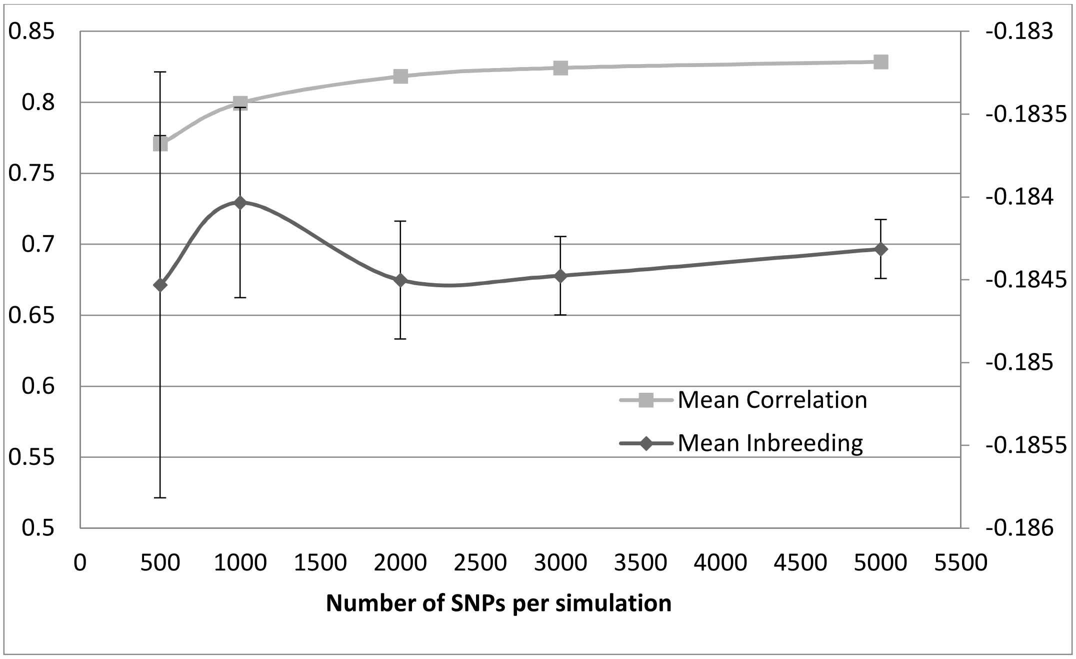


Figure 4.5. Mean correlations and Inbreeding (with 95% confidence intervals) from the bootstrap analysis. The x-axis represents different numbers of linkage equilibrium randomly chosen SNPs in each of the 1000 replicates. Squares shows the mean correlation between the inbreeding measures using the full set (7217 SNPs) and diamonds shows mean inbreeding obtained for each run.

Evaluation of Genetic Relationships Methods

Since the utility of genomic markers gained traction, numerous methods/software packages for measuring genetic similarity have been developed. Thus, the choice of an effective measure of genomic relationships can be challenging and here, we chose the genomic relationships that correlated the most with pedigree relationships. Pair-wise correlations between relationships estimated between those programs are shown in **Figure 4.6**. The KING program, using the Robust estimator, produced the most concordant (r= 0.77, $p < 0.005$) pair-wise relationship estimates to the pedigree relationship coefficients (**Figure 4.3-B**) and therefore we decided to use its estimates in this study. This correlation was surprisingly low given the number of SNPs used. However, it was still within the range of 0.73 and 0. 858 previously reported by Santure *et al.* (2010) and Lopes *et al.* (2013b) respectively. It is worth mentioning that the SNPRelate (MOM) ranked the second with a correlation of (r=0.71, p < 0.005). Although theoretically MLE estimates are more reliable (Zheng *et al.* 2012), SNPRelate (MLE) estimate did not perform as well as their MOM counterparts (r=0.58, p < 0.0005). This may be due a result of the asymptotically unbiased nature of MLE estimates, requiring larger sample sizes to yield accurate estimates.

Genetic Structure and Relationships within the Herd

Relationship measures derived from genomic data are more accurate as they are derived from identity by state sharing of alleles, rather than the probability of sharing by descent predicted from pedigrees (Speed & Balding 2014). Thus, genotype based methods allow observation of between-sibling variation resulting from Mendelian sampling (Lopes *et al.* 2013b). Pedigree estimates of relatedness also assume that founder animals are unrelated (Wang *et al.* (2014) and Speed and Balding (2014)). Yet, this assumption is unrealistic as all individuals are related at some time point in the past (Powell *et al.* 2010). As expected, pair-wise D values were inversely correlated with the pedigree relationships (r= -0.75, p= 2.2e-16). This means that the genetic distance between pairs of individuals became less the higher the pedigree relationships between them and *vise versa*. However, the mean pair-wise D value for these animals was only 0.28 (SD =0.036), which is only slightly higher than the within breed mean of 0.23 (SD=0.009) observed by McCue *et al.* (2012) for Arabian horses. It is remarkable that the two values are this close, given that the animals used in our study belong to the same herd.

The matrix of proportion of alleles identical by state (IBS) between horses can be found in **Table 4.3.** It is a straightforward calculation of the proportion of markers that are identical in genotype between all possible pairs of two individuals. This matrix is useful in finding pairs of individuals that are more similar or different from each other than would be expected by chance in a random homogenous sample. For instance, in the two daughters of the dam ID: 47, daughter ID: 51 is 0.784 similar to her mother, while daughter ID: 50 is 0.773 similar (despite identical pedigree derived relationship values). An interesting observation was that of horse ID: 54 has an overwhelming majority of pair-wise IBS values below 0.65 which is in agreement of the pedigree (**Figure 4.1**). On the other hand, most pair-wise IBS values for horses ID: 59 and ID: 45 are relatively high (> 0.80). This makes sense since they have the largest contribution of progeny in this herd.

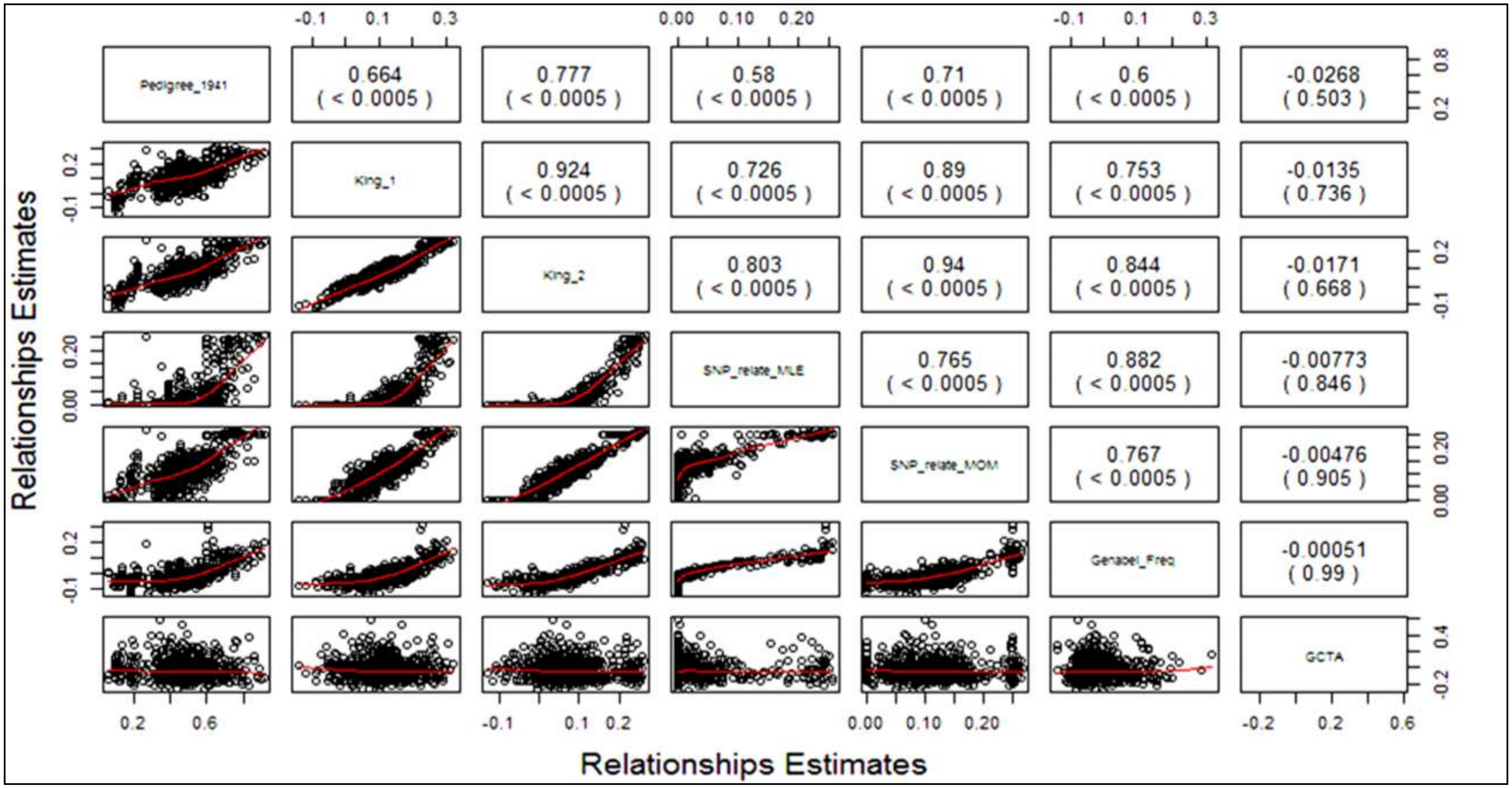


Figure 4.6. Pair-wise correlations between various programs used to estimate relationships and the pedigree estimated relationships. Name of the program is shown in the diagonals. Upper diagonal elements represent correlations (their significance in parenthesis) between relationships measures of various programs. King_1= King (assuming homogenous population, King_2=King (robust estimation), SNP_relate_MLE= SNPRelate (using maximum likelihood estimation), SNP_relate_MOM= SNPRelate (using the method of moments), Genabel=GenABEL (using allele frequencies), GCTA=GCTA (using the default kinship measure).

Table 4.3. Pairwise identity-by-state (IBS) genetic similarity between individuals depicting a higher genome sharing between close relatives. Relatives with the same pedigree based relationships (e.g half sibs) can have different IBS values.

	1642	**1650**	**1658**	**1666**	**1643**	**1651**	**1659**	**1667**	**1644**	**1652**	**1660**	**1668**
1642	1	0.736711	0.724093	0.705788	0.733695	0.736798	0.776804	0.720573	0.784708	0.736784	0.679901	0.71382
1650	0.736711	1	0.73584	0.715398	0.735832	0.759266	0.775801	0.735308	0.709387	0.725515	0.68914	0.757775
1658	0.724093	0.73584	1	0.689189	0.743137	0.739835	0.784592	0.745425	0.732438	0.729329	0.685865	0.740894
1666	0.705788	0.715398	0.689189	1	0.707659	0.731974	0.7349	0.693317	0.744997	0.692229	0.720926	0.684279
1643	0.733695	0.735832	0.743137	0.707659	1	0.723501	0.79562	0.736975	0.751651	0.726125	0.69412	0.743517
1651	0.736798	0.759266	0.739835	0.731974	0.723501	1	0.793517	0.716621	0.717547	0.748594	0.696157	0.725534
1659	0.776804	0.775801	0.784592	0.7349	0.79562	0.793517	1	0.79948	0.775597	0.776814	0.730189	0.80893
1667	0.720573	0.735308	0.745425	0.693317	0.736975	0.716621	0.79948	1	0.719717	0.724602	0.725127	0.756513
1644	0.784708	0.709387	0.732438	0.744997	0.751651	0.717547	0.775597	0.719717	1	0.754314	0.685881	0.729654
1652	0.736784	0.725515	0.729329	0.692229	0.726125	0.748594	0.776814	0.724602	0.754314	1	0.691452	0.722149
1660	0.679901	0.68914	0.685865	0.720926	0.69412	0.696157	0.730189	0.725127	0.685881	0.691452	1	0.672559
1668	0.71382	0.757775	0.740894	0.684279	0.743517	0.725534	0.80893	0.756513	0.729654	0.722149	0.672559	1
1645	0.750642	0.749604	0.753168	0.689848	0.729258	0.747714	0.723186	0.705151	0.747079	0.737703	0.663999	0.737511
1653	0.720954	0.744838	0.758682	0.692265	0.7453	0.767449	0.809637	0.750807	0.756567	0.779892	0.687044	0.752417
1661	0.702355	0.720607	0.721708	0.751714	0.728284	0.740474	0.789925	0.749782	0.679607	0.710519	0.818642	0.699971
1669	0.711867	0.730562	0.724176	0.711249	0.724003	0.706742	0.792994	0.742736	0.741553	0.709116	0.727002	0.741073
1638	0.747304	0.684151	0.681145	0.689297	0.686762	0.69372	0.715053	0.692935	0.805054	0.70327	0.671102	0.687775
1646	0.720858	0.711819	0.683295	0.798127	0.694629	0.723611	0.704413	0.674323	0.79783	0.699202	0.671394	0.680886
1654	0.650729	0.667786	0.656504	0.653846	0.640913	0.652351	0.658664	0.659547	0.647562	0.650682	0.656439	0.636115
1662	0.691145	0.716338	0.689626	0.70678	0.710686	0.718901	0.710183	0.720707	0.718954	0.710171	0.694773	0.797409
1670	0.719027	0.725901	0.731752	0.729294	0.727864	0.74207	0.805958	0.740163	0.734609	0.728231	0.739896	0.751164
1639	0.782438	0.686894	0.694652	0.684894	0.703422	0.702304	0.695168	0.668756	0.789948	0.709008	0.664109	0.68751
1647	0.699265	0.773412	0.689313	0.716249	0.676792	0.784718	0.681232	0.681885	0.663415	0.693793	0.67175	0.688767
1655	0.696959	0.693451	0.786501	0.682635	0.697724	0.704954	0.691494	0.711911	0.709913	0.692873	0.675997	0.694772
1663	0.714445	0.696021	0.703401	0.688509	0.69368	0.714745	0.710909	0.758656	0.702858	0.686562	0.679718	0.728373
1671	0.701991	0.707804	0.681194	0.739385	0.686388	0.69718	0.732244	0.68915	0.724729	0.677464	0.796736	0.705534
1640	0.740213	0.702459	0.722392	0.680935	0.70889	0.711893	0.792645	0.740185	0.766492	0.721936	0.673379	0.71725
1648	0.691233	0.677635	0.698305	0.67749	0.692166	0.71479	0.686957	0.678292	0.750717	0.804032	0.646285	0.689497
1656	0.670702	0.688007	0.672739	0.716143	0.659576	0.688607	0.678392	0.673209	0.70523	0.659665	0.664834	0.661252
1664	0.672999	0.675512	0.681271	0.757433	0.67774	0.684027	0.7038	0.688445	0.702759	0.668956	0.760451	0.684159
1672	0.723669	0.715098	0.696069	0.796208	0.710775	0.719488	0.765805	0.705622	0.744285	0.698002	0.71323	0.688469
1641	0.734571	0.721567	0.718915	0.704328	0.787607	0.711574	0.719835	0.707154	0.744842	0.700859	0.676867	0.721002
1649	0.682255	0.687724	0.685218	0.691074	0.684576	0.689292	0.681884	0.65912	0.710679	0.692119	0.670789	0.68834
1657	0.705482	0.68722	0.690513	0.724346	0.685109	0.690532	0.705043	0.6774	0.72598	0.697204	0.675529	0.673477
1665	0.693672	0.689144	0.680868	0.762589	0.675278	0.6956	0.707997	0.671609	0.717367	0.682547	0.763725	0.657452
1673	0.719858	0.720014	0.704515	0.715328	0.723227	0.733578	0.792152	0.767198	0.719372	0.71853	0.701355	0.734562

Table 4.3 (Continued)

	1645	1653	1661	1669	1638	1646	1654	1662	1670	1639	1647	1655
1642	0.750642	0.720954	0.702355	0.711867	0.747304	0.720858	0.650729	0.691145	0.719027	0.782438	0.699265	0.696959
1650	0.749604	0.744838	0.720607	0.730562	0.684151	0.711819	0.667786	0.716338	0.725901	0.686894	0.773412	0.693451
1658	0.753168	0.758682	0.721708	0.724176	0.681145	0.683295	0.656504	0.689626	0.731752	0.694652	0.689313	0.786501
1666	0.689848	0.692265	0.751714	0.711249	0.689297	0.798127	0.653846	0.70678	0.729294	0.684894	0.716249	0.682635
1643	0.729258	0.7453	0.728284	0.724003	0.686762	0.694629	0.640913	0.710686	0.727864	0.703422	0.676792	0.697724
1651	0.747714	0.767449	0.740474	0.706742	0.69372	0.723611	0.652351	0.718901	0.74207	0.702304	0.784718	0.704954
1659	0.723186	0.809637	0.789925	0.792994	0.715053	0.704413	0.658664	0.710183	0.805958	0.695168	0.681232	0.691494
1667	0.705151	0.750807	0.749782	0.742736	0.692935	0.674323	0.659547	0.720707	0.740163	0.668756	0.681885	0.711911
1644	0.747079	0.756567	0.679607	0.741553	0.805054	0.79783	0.647562	0.718954	0.734609	0.789948	0.663415	0.709913
1652	0.737703	0.779892	0.710519	0.709116	0.70327	0.699202	0.650682	0.710171	0.728231	0.709008	0.693793	0.692873
1660	0.663999	0.687044	0.818642	0.727002	0.671102	0.671394	0.656439	0.694773	0.739896	0.664109	0.67175	0.675997
1668	0.737511	0.752417	0.699971	0.741073	0.687775	0.680886	0.636115	0.797409	0.751164	0.68751	0.688767	0.694772
1645	1	0.760905	0.666885	0.714546	0.699495	0.750409	0.647072	0.784323	0.720396	0.781751	0.793015	0.777446
1653	0.760905	1	0.725755	0.716854	0.703281	0.699959	0.654548	0.712395	0.744054	0.70312	0.720752	0.728102
1661	0.666885	0.725755	1	0.706659	0.66353	0.65841	0.671323	0.698752	0.735699	0.645757	0.701161	0.692274
1669	0.714546	0.716854	0.706659	1	0.695172	0.73238	0.639852	0.707634	0.817271	0.689302	0.682552	0.673932
1638	0.699495	0.703281	0.66353	0.695172	1	0.726003	0.652447	0.690553	0.700584	0.799293	0.664337	0.676375
1646	0.750409	0.699959	0.65841	0.73238	0.726003	1	0.656376	0.727996	0.727217	0.735535	0.738562	0.688367
1654	0.647072	0.654548	0.671323	0.639852	0.652447	0.656376	1	0.651311	0.631138	0.635363	0.67285	0.675008
1662	0.784323	0.712395	0.698752	0.707634	0.690553	0.727996	0.651311	1	0.706081	0.723829	0.745064	0.73216
1670	0.720396	0.744054	0.735699	0.817271	0.700584	0.727217	0.631138	0.706081	1	0.68728	0.690822	0.672031
1639	0.781751	0.70312	0.645757	0.689302	0.799293	0.735535	0.635363	0.723829	0.68728	1	0.697278	0.718465
1647	0.793015	0.720752	0.701161	0.682552	0.664337	0.738562	0.67285	0.745064	0.690822	0.697278	1	0.7294
1655	0.777446	0.728102	0.692274	0.673932	0.676375	0.688367	0.675008	0.73216	0.672031	0.718465	0.7294	1
1663	0.786266	0.712123	0.685586	0.69936	0.686158	0.696381	0.657883	0.764323	0.702135	0.709274	0.736172	0.747965
1671	0.695977	0.688876	0.729934	0.738137	0.704227	0.752305	0.644701	0.684219	0.745592	0.697798	0.709696	0.673666
1640	0.673933	0.750417	0.708622	0.717437	0.799296	0.688868	0.644394	0.660044	0.730335	0.719579	0.650272	0.657141
1648	0.790385	0.797743	0.64687	0.666592	0.703775	0.713761	0.651852	0.708178	0.67364	0.7195	0.731881	0.718825
1656	0.688928	0.688511	0.658309	0.691092	0.670469	0.760076	0.77263	0.682772	0.685598	0.662971	0.728678	0.682361
1664	0.687491	0.682919	0.692816	0.787813	0.686027	0.760565	0.634468	0.692308	0.801053	0.673913	0.704792	0.676072
1672	0.692301	0.712448	0.740627	0.73682	0.698464	0.777816	0.647018	0.697533	0.730246	0.703444	0.705388	0.682475
1641	0.798481	0.718509	0.680553	0.704061	0.723745	0.729045	0.647527	0.741958	0.700857	0.76856	0.73246	0.724246
1649	0.762796	0.690484	0.665721	0.666561	0.676151	0.713763	0.625184	0.718662	0.661204	0.718858	0.715706	0.68749
1657	0.702953	0.691576	0.669409	0.713229	0.692196	0.773155	0.770731	0.703201	0.692044	0.684949	0.712878	0.694385
1665	0.694938	0.69441	0.719147	0.706961	0.690923	0.781399	0.654612	0.683307	0.729832	0.680689	0.728114	0.683483
1673	0.716896	0.745312	0.758854	0.709411	0.667062	0.711948	0.647029	0.734166	0.718168	0.682072	0.701456	0.690919

Table 4.3 (Continued)

	1663	1671	1640	1648	1656	1664	1672	1641	1649	1657	1665	1673
1642	0.714445	0.701991	0.740213	0.691233	0.670702	0.672999	0.723669	0.734571	0.682255	0.705482	0.693672	0.719858
1650	0.696021	0.707804	0.702459	0.677635	0.688007	0.675512	0.715098	0.721567	0.687724	0.68722	0.689144	0.720014
1658	0.703401	0.681194	0.722392	0.698305	0.672739	0.681271	0.696069	0.718915	0.685218	0.690513	0.680868	0.704515
1666	0.688509	0.739385	0.680935	0.67749	0.716143	0.757433	0.796208	0.704328	0.691074	0.724346	0.762589	0.715328
1643	0.69368	0.686388	0.70889	0.692166	0.659576	0.67774	0.710775	0.787607	0.684576	0.685109	0.675278	0.723227
1651	0.714745	0.69718	0.711893	0.71479	0.688607	0.684027	0.719488	0.711574	0.689292	0.690532	0.6956	0.733578
1659	0.710909	0.732244	0.792645	0.686957	0.678392	0.7038	0.765805	0.719835	0.681884	0.705043	0.707997	0.792152
1667	0.758656	0.68915	0.740185	0.678292	0.673209	0.688445	0.705622	0.707154	0.65912	0.6774	0.671609	0.767198
1644	0.702858	0.724729	0.766492	0.750717	0.70523	0.702759	0.744285	0.744842	0.710679	0.72598	0.717367	0.719372
1652	0.686562	0.677464	0.721936	0.804032	0.659665	0.668956	0.698002	0.700859	0.692119	0.697204	0.682547	0.71853
1660	0.679718	0.796736	0.673379	0.646285	0.664834	0.760451	0.71323	0.676867	0.670789	0.675529	0.763725	0.701355
1668	0.728373	0.705534	0.71725	0.689497	0.661252	0.684159	0.688469	0.721002	0.68834	0.673477	0.657452	0.734562
1645	0.786266	0.695977	0.673933	0.790385	0.688928	0.687491	0.692301	0.798481	0.762796	0.702953	0.694938	0.716896
1653	0.712123	0.688876	0.750417	0.797743	0.688511	0.682919	0.712448	0.718509	0.690484	0.691576	0.69441	0.745312
1661	0.685586	0.729934	0.708622	0.64687	0.658309	0.692816	0.740627	0.680553	0.665721	0.669409	0.719147	0.758854
1669	0.69936	0.738137	0.717437	0.666592	0.691092	0.787813	0.73682	0.704061	0.666561	0.713229	0.706961	0.709411
1638	0.686158	0.704227	0.799296	0.703775	0.670469	0.686027	0.698464	0.723745	0.676151	0.692196	0.690923	0.667062
1646	0.696381	0.752305	0.688868	0.713761	0.760076	0.760565	0.777816	0.729045	0.713763	0.773155	0.781399	0.711948
1654	0.657883	0.644701	0.644394	0.651852	0.77263	0.634468	0.647018	0.647527	0.625184	0.770731	0.654612	0.647029
1662	0.764323	0.684219	0.660044	0.708178	0.682772	0.692308	0.697533	0.741958	0.718662	0.703201	0.683307	0.734166
1670	0.702135	0.745592	0.730335	0.67364	0.685598	0.801053	0.730246	0.700857	0.661204	0.692044	0.729832	0.718168
1639	0.709274	0.697798	0.719579	0.7195	0.662971	0.673913	0.703444	0.76856	0.718858	0.684949	0.680689	0.682072
1647	0.736172	0.709696	0.650272	0.731881	0.728678	0.704792	0.705388	0.73246	0.715706	0.712878	0.728114	0.701456
1655	0.747965	0.673666	0.657141	0.718825	0.682361	0.676072	0.682475	0.724246	0.68749	0.694385	0.683483	0.690919
1663	1	0.694846	0.668531	0.704164	0.68163	0.684833	0.683239	0.71364	0.704703	0.685027	0.676695	0.780831
1671	0.694846	1	0.685168	0.656926	0.702082	0.784203	0.771212	0.70695	0.689232	0.705594	0.784545	0.684535
1640	0.668531	0.685168	1	0.695312	0.67314	0.68305	0.72291	0.703891	0.680641	0.683857	0.686465	0.71206
1648	0.704164	0.656926	0.695312	1	0.693073	0.68912	0.679922	0.730119	0.742156	0.707904	0.693936	0.69524
1656	0.68163	0.702082	0.67314	0.693073	1	0.727086	0.722232	0.69437	0.675958	0.808449	0.731535	0.689223
1664	0.684833	0.784203	0.68305	0.68912	0.727086	1	0.754434	0.708833	0.67874	0.726731	0.805432	0.698004
1672	0.683239	0.771212	0.72291	0.679922	0.722232	0.754434	1	0.71201	0.70444	0.737691	0.767972	0.747631
1641	0.71364	0.70695	0.703891	0.730119	0.69437	0.708833	0.71201	1	0.742747	0.717135	0.710518	0.718268
1649	0.704703	0.689232	0.680641	0.742156	0.675958	0.67874	0.70444	0.742747	1	0.683632	0.689575	0.694717
1657	0.685027	0.705594	0.683857	0.707904	0.808449	0.726731	0.737691	0.717135	0.683632	1	0.737163	0.70748
1665	0.676695	0.784545	0.686465	0.693936	0.731535	0.805432	0.767972	0.710518	0.689575	0.737163	1	0.710625
1673	0.780831	0.684535	0.71206	0.69524	0.689223	0.698004	0.747631	0.718268	0.694717	0.70748	0.710625	1

The within herd MDS analysis (**Figure 4.7-A and 4.7-B**) allows consideration of components of variation from across the entire herd, rather than only between pairs of animals. Therefore, horses with similar genetic makeup cluster together in multidimensional space. The MDS analysis demonstrates the uniqueness of horse ID:54 which is clearly separated from the herd. The within herd STRUCTURE analysis showed that the best K value is K=5, which is approximately equal to the number of families in the herd. The STRUCTURE analysis (**Figure 4.8-a**) supports the MDS analysis on horse ID:54 and assigns her and her daughters to the same sub-group membership, which agrees with the reported recent addition of this individual to the herd. The MDS analysis also confirms the high degree of relatedness of horses ID: 59 and ID: 45 to a many members of this group. STRUCTURE analysis also detected this relationship, depicting ID: 59 and 45 as ancestors in the herd with many individuals sharing their ancestry. The STRUCTURE analysis also accurately captured parent-offspring relationships. For example, parent ID: 38 and offspring ID: 39 as well as parents ID: 59 and ID: 64 and their offspring ID: 69 and ID: 70. Each of these methods demonstrates the power and utility of SNPs as a tool for reconstructing unknown, prehistoric or erroneous pedigree relationships.

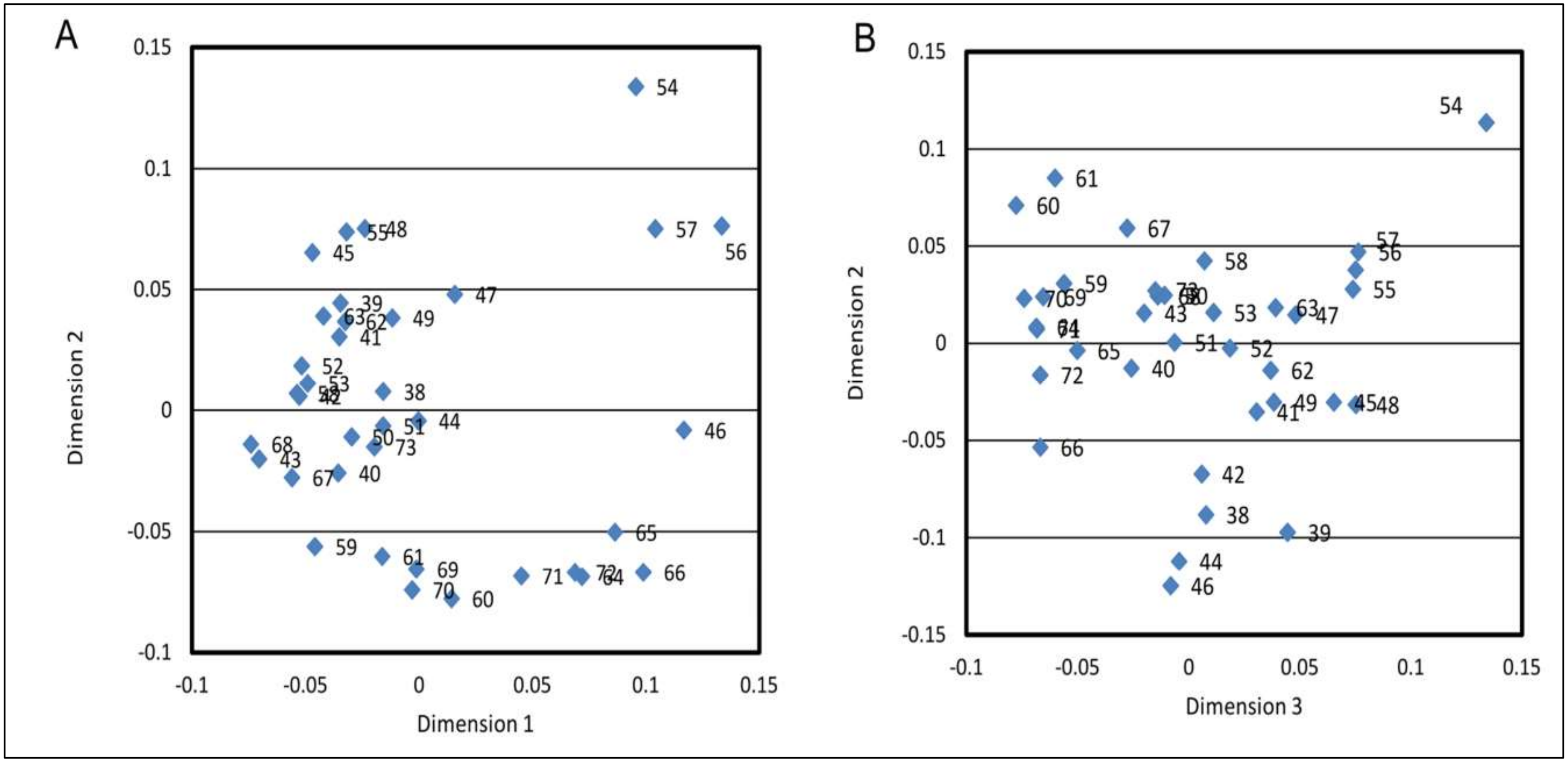
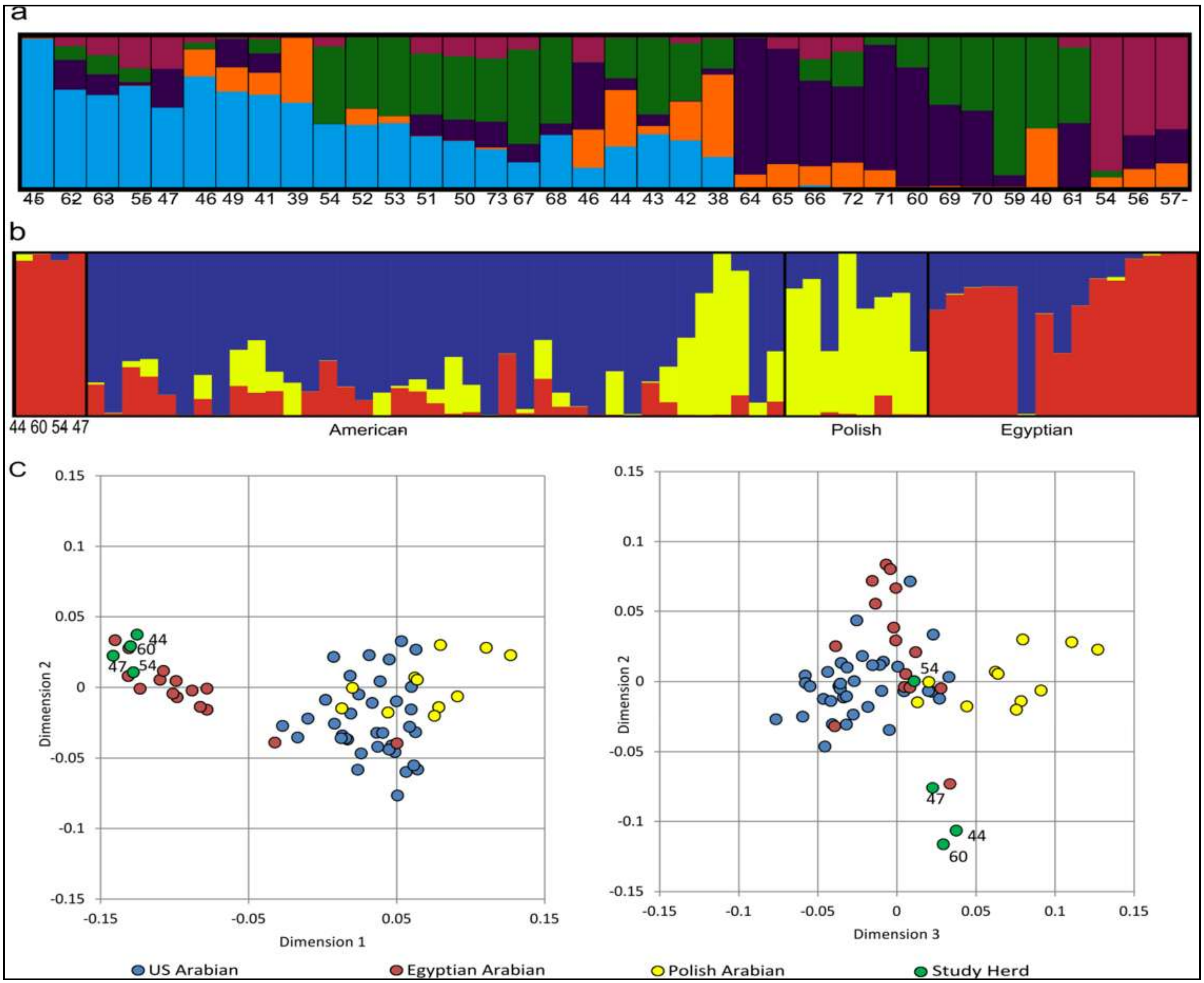


Figure 4.7. Within herd multidimensional scaling analysis of genotypes. **A.** Dimension one vs two right **B.** Dimension two vs three.

Genetic Similarity of the Herd to Other Arabian Horses

As a whole, this herd of horses is markedly genetically similar to the reference Arabian horses of Egyptian origin. The founders of this herd are clearly clustered by the MDS analysis within the Egyptian Arabians (**Figure 4.8-c)**. The extension of this analysis (dimension two vs three) adds detail, separating horse ID: 54 from the other founder animals of this herd and closer to the other Egyptian and US Arabians. The STRUCTURE analysis comparing the herd to other Arabian horses also supported their reported Egyptian ancestry (**Figure 4.8-b**). The herd's grandparents share ancestry with most of the Egyptian Arabian reference individuals (shown in brown). The best K value of 3 was expected since we had Arabian horse belonging to 3 distinct origins (American, Polish and Egyptian Arabian horses). Differences among the founders are also apparent. Horse ID: 44, for example, possesses a 4 % proportion of his ancestry in common with polish bloodlines (shown in yellow). Also, horse ID: 54 has some 4.2 % similarity to the reference US-registered horses (as detected by the MDS analysis, **Figure 4.8-c**). The results also showed that an individual identified as Egyptian Arabian had a striking similarity of 99% to US Arabian horses. This finding was also supported by the MDS analysis (**Figure 4.8-c**) where the horse clusters with US Arabian horses. Given the evidence from all aforementioned analyses, we presume that this may be a result of misidentification of the horse as such a finding is difficult to have arisen due to chance alone. Altogether, the three types of population genetic analyses strongly illustrate the Egyptian Arabian ancestry of the herd.

Figure 4.8. a. Population of origin assignments using STRUCTURE for the animals with the group to each of the five clusters. Different colors indicate different assignment combinations (proportions of membership). Animals' IDs are shown in the labels below the figure. **b.** Population of origin assignments using STRUCTURE for the group's founders amongst other Arabian horses to each of the three clusters. Actual population of origin for each individual is shown in labels below the figure. Proportion of membership is colored in Blue for US Arabian, yellow for Polish Arabian and brown for Egyptian Arabian horses. **c.** Multidimensional scaling of the group's founders amongst other Arabian horses. Left figure is dimension one vs two and right figure is dimension two vs three.


a
45 62 63 55 47 46 49 41 39 54 52 53 51 50 73 67 68 46 44 43 42 38 64 65 66 72 71 60 69 70 59 40 61 54 56 57-
b
44 60 54 47
American
Polish
Egyptian
C
0.15
0.1
0.05
0
-0.05
-0.1
-0.15
Dimeension 2
44
60
47
54
-0.15
-0.05
0.05
0.15
Dimension 1
0.15
0.1
0.05
0
-0.05
-0.1
-0.15
Dimension 2
54
47
44
60
-0.15
-0.1
-0.05
0
0.05
0.1
0.15
Dimension 3
US Arabian
Egyptian Arabian
Polish Arabian
Study Herd

CONCLUSION

The purpose of this study was to elucidate the genetic background of the herd using modern high-throughput genotyping techniques. Estimates of relatedness within the herd and amongst the herd founders and other Arabian Horses highlighted some key differences from pedigree inferred relationships and historical data. Additionally, analysis based on genotypes enabled re-capitulation of the familial relationships among these horses and demonstrated their Egyptian ancestry among other Arabian horse bloodlines. Altogether, our results signify the practical benefits of genome-wide genotyping methods for quantification of inbreeding and inference of relationships beyond those illustrated in the pedigree.

REFERENCES

Ai H., Huang L. & Ren J. (2013) Genetic diversity, linkage disequilibrium and selection signatures in chinese and Western pigs revealed by genome-wide SNP markers. *Plos One* **8**, e56001-e.

Aulchenko Y.S., Ripke S., Isaacs A. & van Duijn C.M. (2007) GenABEL: an R library for genome-wide association analysis. *Bioinformatics (Oxford, England)* **23**, 1294-6.

Binns M.M., Boehler D.a., Bailey E., Lear T.L., Cardwell J.M. & Lambert D.H. (2012) Inbreeding in the Thoroughbred horse. *Animal genetics* **43**, 340-2.

Bower M.A., McGivney B.A., Campana M.G., Gu J., Andersson L.S., Barrett E., Davis C.R., Mikko S., Stock F., Voronkova V., Bradley D.G., Fahey A.G., Lindgren G., MacHugh D.E., Sulimova G. & Hill E.W. (2012) The genetic origin and history of speed in the Thoroughbred racehorse. *Nature communications* **3**, 643.

Brooks S.a., Gabreski N., Miller D., Brisbin A., Brown H.E., Streeter C., Mezey J., Cook D. & Antczak D.F. (2010) Whole-genome SNP association in the horse: identification of a deletion in myosin Va responsible for Lavender Foal Syndrome. *PLoS genetics* **6**, e1000909-e.

Cervantes I., Gutiérrez J.P., Molina A., Goyache F. & Valera M. (2009) Genealogical analyses in open populations: the case of three Arab-derived Spanish horse breeds. *Journal of animal breeding and genetics = Zeitschrift für Tierzüchtung und Züchtungsbiologie* **126**, 335-47.

Colleau J.-J., . (2002) An indirect approach to the extensive calculation of relationship coefficients. *Genetics, selection, evolution : GSE* **34**, 409-21.

Cook D., Gallagher P.C. & Bailey E. (2010) Genetics of swayback in American Saddlebred horses. *Animal genetics* **41 Suppl 2**, 64-71.

Evanno G., Regnaut S. & Goudet J. (2005) Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. *Molecular ecology* **14**, 2611-20.

Jakobsson M. & Rosenberg N.A. (2007) CLUMPP: a cluster matching and permutation program for dealing with label switching and multimodality in analysis of population structure. *Bioinformatics (Oxford, England)* **23**, 1801-6.

Jun J., Cho Y., Hu H., Kim H.-M., Jho S., Gadhvi P., Park K., Lim J., Paek W., Han K., Manica A., Edwards J.S. & Bhak J. (2014) Whole genome sequence and analysis of the Marwari horse breed and its genetic origin. *BMC Genomics* **15**, S4-S.

Lopes M., Silva F., Harlizius B., Duijvesteijn N., Lopes P., Guimaraes S. & Knol E. (2013a) Improved estimation of inbreeding and kinship in pigs using optimized SNP panels. *BMC genetics* **14**, 92.

Lopes M.S., Silva F.F., Harlizius B., Duijvesteijn N., Lopes P.S., Guimarães S.E. & Knol E.F. (2013b) Improved estimation of inbreeding and kinship in pigs using optimized SNP panels. *BMC genetics* **14**, 92.

Lutaaya B.E., Misztal I., Bertrand J.K. & Mabry J.W. (1999) Inbreeding in populations with incomplete pedigrees. *Journal of Animal Breeding and Genetics* **116**, 475-80.

MacCluer J.W., Boyce A.J., Dyke B., Weitkamp L.R., Pfenning D.W. & Parsons C.J. (1983) Inbreeding and pedigree structure in Standardbred horses. *J. Hered.* **74**, 394-9.

Manichaikul A., Mychaleckyj J.C., Rich S.S., Daly K., Sale M. & Chen W.-M. (2010) Robust relationship inference in genome-wide association studies. *Bioinformatics (Oxford, England)* **26**, 2867-73.

McCue M.E., Bannasch D.L., Petersen J.L., Gurr J., Bailey E., Binns M.M., Distl O., Guérin G., Hasegawa T., Hill E.W., Leeb T., Lindgren G., Penedo M.C.T., Røed K.H., Ryder O.a., Swinburne J.E., Tozaki T., Valberg S.J., Vaudin M., Lindblad-Toh K., Wade C.M. & Mickelson J.R. (2012) A high density SNP array for the domestic horse and extant Perissodactyla: utility for association mapping, genetic diversity, and phylogeny studies. *PLoS genetics* **8**, e1002451-e.

Moureaux S., Verrier É., Ricard A. & Mériaux J.C. (1996) Genetic variability within French race and riding horse breeds from genealogical data and blood marker polymorphisms. *Genetics Selection Evolution* **28**, 83.

Mucha S. & Windig J.J. (2009) Effects of incomplete pedigree on genetic management of the Dutch Landrace goat. *Journal of Animal Breeding and Genetics* **126**, 250-6.

Pirault P., Danvy S., Verrier E. & Leroy G. (2013) Genetic Structure and Gene Flows within Horses: A Genealogical Study at the French Population Scale. *PLoS ONE* **8**, e61544.

Porter V M.L. (2002) *A world dictionary of livestock breeds, types and varieties*. Wallingford: CABI Publishing.

Powell J.E., Visscher P.M. & Goddard M.E. (2010) Reconciling the analysis of IBD and IBS in complex trait studies. *Nature reviews. Genetics* **11**, 800-5.

Pritchard J.K., Stephens M. & Donnelly P. (2000) Inference of population structure using multilocus genotype data. *Genetics* **155**, 945-59.

Purcell S., Neale B., Todd-Brown K., Thomas L., Ferreira Manuel A R., Bender D., Maller J., Sklar P., de Bakker Paul I W., Daly Mark J. & Sham Pak C. (2007) PLINK: A Tool Set for Whole-Genome Association and Population-Based Linkage Analyses. *American journal of human genetics* **81**, 559-75.

Rolf M.M., Taylor J.F., Schnabel R.D., McKay S.D., McClure M.C., Northcutt S.L., Kerley M.S. & Weaber R.L. (2010) Impact of reduced marker set estimation of genomic relationship matrices on genomic selection for feed efficiency in Angus cattle. *BMC Genetics* **11**, 24.

Santure A.W., Stapley J., Ball A.D., Birkhead T.R., Burke T. & Slate J. (2010) On the use of large marker panels to estimate inbreeding and relatedness: empirical and simulation studies of a pedigreed zebra finch population typed at 771 SNPs. *Molecular ecology* **19**, 1439-51.

Sargolzaei M., Iwaisaki H. & Colleau J.J. (2006) CFC: a tool for monitoring genetic diversity. pp. 27-8. Instituto Prociência.

Speed D. & Balding D.J. (2014) Relatedness in the post-genomic era: is it still useful? *Nature Reviews Genetics* **16**, 33-44.

VanRaden P.M. (2008) Efficient methods to compute genomic predictions. *Journal of Dairy Science* **91**, 4414-23.

VanRaden P.M., Olson K.M., Wiggans G.R., Cole J.B. & Tooker M.E. (2011) Genomic inbreeding and relationships among Holsteins, Jerseys, and Brown Swiss. *Journal of Dairy Science* **94**, 5673-82.

Wang H., Misztal I. & Legarra A. (2014) Differences between genomic-based and pedigree-based relationships in a chicken population, as a function of quality control and pedigree links among individuals. *Journal of animal breeding and genetics = Zeitschrift für Tierzüchtung und Züchtungsbiologie* **131**, 445-51.

Wiler R., Leber R., Moore B.B., VanDyk L.F., Perryman L.E. & Meek K. (1995) Equine severe combined immunodeficiency: a defect in V(D)J recombination and DNA-dependent protein kinase activity. *Proceedings of the National Academy of Sciences* **92**, 11485-9.

Yang J., Lee S.H., Goddard M.E. & Visscher P.M. (2011) GCTA: a tool for genome-wide complex trait analysis. *Am J Hum Genet* **88**, 76-82.

Zheng X., Levine D., Shen J., Gogarten S.M., Laurie C. & Weir B.S. (2012) A high-performance computing toolset for relatedness and principal component analysis of SNP data. *Bioinformatics (Oxford, England)* **28**, 3326-8.

CHAPTER 5

SUMMARY

The recent improvements in the methods of whole genome sequencing and genotyping have largely benefitted the horse genetics community as well as other livestock and non-model organisms. These improvements coincided with a rapid development of tools and applications as well as in computational power available to analyze the relatively large amount of data resulting from sequencing and genotyping projects.

Following completion of the horse genome in 2009, a plethora of studies have utilized the sequence information. The SNPs identified as part of the equine genome project helped design the 50K and later 70K Illumina equine genotyping array. These genotyping arrays assisted in the characterization of the phylogenetic, diversity and inbreeding measures in a large number of horse breeds (Binns *et al.* 2012; McCue *et al.* 2012; Petersen *et al.* 2013). Additionally, these genotyping arrays enabled mapping a number of important morphological and health traits in the horse (Brooks *et al.* 2010; Makvandi-Nejad *et al.* 2012). The falling cost of next generation sequencing technology resulted in generation of a number of sequences for several breeds of horse (Doan *et al.* 2012; Jun *et al.* 2014). To date these projects have added to the known variants for each of those breeds.

In chapter two of the thesis, variants discovered by next generation paired-end sequencing technology generated were annotated in six genomes belonging horses from six different breeds. This technology proved to be very successful recently in genome annotation and variants discovery in cattle and chicken (Daetwyler *et al.* 2014; Yan *et al.* 2014). The functional Annotation of Animal Genomes (FAANG) project, an international efforts in annotating various livestock genomes is primarily utilizing next generation sequencing technologies to achieve its objectives (Andersson *et al.* 2015). The work in this dissertation adds to the growing wealth of genomic data made available for the horse. We detected and functionally annotated 8,128,658 SNPs and 830,370 small INDELs. Of the SNPs we detected, 5,221,242 SNPs were novel SNPs not reported previously in ENSEMBL or dbSNP data bases. Additionally, we were able to detect structural and copy number variations unique to each of six horses that we also functionally annotated. Incorporating a larger number of breeds than previous studies enabled us to discover a larger number of variants of all types. It also enabled us to determine the private variants pertaining to each horse which is informative of the specific biology of these horse breeds. These variations are a valuable addition to the existing genomic variation in the horse. We formatted them into user friendly tracks and are now available publically on-line for the horse genetics community for future studies. These annotations will be utilized in the construction of SNPs and INDELs marker panels as well as in the creation of high density linkage maps. In addition, our analysis revealed a copy number gain at the latherin locus (*LATH*) in all six horses but the magnitude of the gain was different between different horses. Latherin is a surface-active, non-glycosylated protein that is hypothesized to play an important role in the thermoregulation of the horse. It is presumed to do that by acting as a wetting agent that facilitates evaporative cooling by reducing water surface tension at low concentrations (McDonald *et al.* 2009).

In chapter three, a mixed model based GWAS study was used to identify QTLs contributing to the variation in height at the withers in horses. Mapping QTLs in the horse was largely based on traditional methods such as linkage mapping. However, the availability of genome-wide genotyping arrays revolutionized the QTL mapping studies by making available thousands of SNPs across the genome that were used in GWAS studies to map QTLs. However, a hurdle that was always of concern when conducting a GWAS was the existence of confounding factors such as kinship and population structure which results in false positive results. Mixed models are very powerful in accounting for both these factors in GWAS studies and are now considered the gold standard method for that purpose (Hoffman 2013). Unlike most GWAS studies which assume an additive model, we used a dominant model to map the QTLs which enabled us to discover QTLs affecting withers height dominantly. To complement GWAS, a cross-population composite likelihood ratio test (XP-CLR) test was applied in order to search for regions under selection in the genomes of horses of large versus small skeletal size. Both the GWAS and the XP-CLR test detected a significant locus at ECA1: 37676322 in an intron of the *ANKRD1* gene. *ANKRD1* is involved in the signaling pathways of muscle remodeling and differentiation which is suggestive of the role it might be playing in influencing withers height variation between horses. We were able to verify our finding by genotyping an independent sample of 90 American Miniature horses. Our results showed that horses possessing the GG or AG genotypes were 4.064 cm taller than those with the AA genotype.

Chapter four of this thesis demonstrates the utility of the Equine SNP70 genotyping array in assessing relatedness, inbreeding and genetic structure in an Arabian horse herd. Traditionally, the genetic structure was assessed using microsatalite markers (Khanshour *et al.* 2013) and inbreeding was calculated using pedigree information (Pirault *et al.* 2013). The Equine SNP70 genotyping array provided a very powerful alternative to microsatellite markers in assessing inbreeding and genetic structure. Estimates of relatedness within the herd and amongst the herd founders and other Arabian Horses highlighted some key differences from pedigree relationships. Moreover, the analysis based on the SNP70 genotyping array enabled re-capitulation of the familial relationships among these horses and demonstrated their Egyptian ancestry among other Arabian horse bloodlines.

During the past decade, a remarkable improvement has taking place in the cost, speed and read length of NGS technology. The ever increasing technological advancement will result in even longer reads at a lower sequencing cost. For instance PacBio RS II system has a mean of 20 kilobases for a very competitive price making it an ideal tool to finish genome assemblies. Nevertheless, errors during sample preparation or sequencing are still a limiting factor in NGS technologies. Sequencing errors could be the result of amplification bias during PCR, polymerase mistakes. These errors especially impact the ability to detect of rare variants (Edward J Fox 2014). Therefore, future research needs to focus at improving the accuracy of base calling in different NGS technologies. Future NGS platforms should also ideally be affordable, fast, and have high accuracy. In addition, more advancement needs to be made both in computational algorithms and molecular genotyping in detecting SNVs and SVs using NGS reads. More specifically, more improvement in the boundaries of SVs and CNVs events and more precise quantification of CNVs is required (Handsaker *et al.* 2015). New technologies such as droplet digital PCR (ddPCR) proved to be very accurate in determining copy number variation (with a concordance rate of 99.9%) although its cost is still a limiting factor. That been said, NGS is very promising in revolutionizing genetic diagnostics, prognostics, disease association and clinical microbiology (Salipante S. J. 2013; Lohmann & Klein 2014). An excellent example of the application of NGS technology in diagnostics is the development of a NGS test for mutations *BRCA1* and *BRCA2* genes implicated in the development of breast cancer in humans (Feliubadaló *et al.* 2012). Also, in horses, NGS have proved successful for discovery and screening of causal variants in rare genetic disorders such as Incontinentia Pigmenti (Towers *et al.* 2013).

Being a non-model organism, variants in the equine genome are still poorly characterized. The work presented in this thesis utilizes the recent advancements in sequencing and genotyping technology and forms the basis for many future research studies. The NGS variants annotated in this work could be utilized in the discovery of causal mutations for various production and health traits in the horse. Moreover, these variants are a valuable asset to fully exploit the similarity between the equine and human genomes in human biomedical research. In addition, the work presented here utilized the equine Equine SNP50 and Equine SNP70 genotyping arrays for QTL mapping and characterizing the genomic inbreeding of an Arabian horse herd respectively. Therefore, the findings discussed in this dissertation can be utilized by equine breeders, clinicians and researchers in future studies.

REFERENCES

Andersson L., Archibald A.L., Bottema C.D., Brauning R., Burgess S.C., Burt D.W., Casas E., Cheng H.H., Clarke L., Couldrey C., Dalrymple B.P., Elsik C.G., Foissac S., Giuffra E., Groenen M.A., Hayes B.J., Huang L.S., Khatib H., Kijas J.W. & Kim H. (2015) Coordinated international action to accelerate genome-to-phenome with FAANG, the Functional Annotation of Animal Genomes project. *Genome Biology* **16**, 57.

Binns M.M., Boehler D.a., Bailey E., Lear T.L., Cardwell J.M. & Lambert D.H. (2012) Inbreeding in the Thoroughbred horse. *Animal genetics* **43**, 340-2.

Brooks S.A., Gabreski N., Miller D., Brisbin A., Brown H.E., Streeter C., Mezey J., Cook D. & Antczak D.F. (2010) Whole-genome SNP association in the horse: identification of a deletion in myosin Va responsible for Lavender Foal Syndrome. *PLoS genetics* **6**, e1000909-e.

Daetwyler H.D., Capitan A., Pausch H., Stothard P., Binsbergen R.v., Brøndum R.F., Liao X., Djari A., Rodriguez S.C., Grohs C., Esquerré D., Bouchez O., Rossignol M.-N., Klopp C., Rocha D., Fritz S., Eggen A., Bowman P.J., Coote D., Chamberlain A.J., Anderson C., VanTassell C.P., Hulsegge I., Goddard M.E., Guldbrandtsen B., Lund M.S., Veerkamp R.F., Boichard D.A., Fries R. & Hayes B.J. (2014) Whole-genome sequencing of 234 bulls facilitates mapping of monogenic and complex traits in cattle. *Nature Genetics* **46**, 858-65.

Doan R., Cohen N.D., Sawyer J., Ghaffari N., Johnson C.D. & Dindot S.V. (2012) Whole-genome sequencing and genetic variant analysis of a Quarter Horse mare. *BMC Genomics* **13**, 78-.

Edward J Fox K.S.R.-B., Mary J Emond and Lawrence A Loeb (2014) Accuracy of Next Generation Sequencing Platforms. *Journal of Next Generation: Sequencing & Applications* **2014**.

Feliubadaló L., Lopez-Doriga A., Castellsagué E., Valle J.d., Menéndez M., Tornero E., Montes

E., Cuesta R., Gómez C., Campos O., Pineda M., González S., Moreno V., Brunet J., Blanco I., Serra E., Capellá G. & Lázaro C. (2012) Next-generation sequencing meets genetic diagnostics: development of a comprehensive workflow for the analysis of BRCA1 and BRCA2 genes. *European Journal of Human Genetics* **21**, 864-70.

Handsaker R.E., Doren V.V., Berman J.R., Genovese G., Kashin S., Boettger L.M. & McCarroll S.A. (2015) Large multiallelic copy number variations in humans. *Nature Genetics* **47**, 296-303.

Hoffman G.E. (2013) Correcting for Population Structure and Kinship Using the Linear Mixed Model: Theory and Extensions. *Plos One* **8**, e75707-e.

Jun J., Cho Y., Hu H., Kim H.-M., Jho S., Gadhvi P., Park K., Lim J., Paek W., Han K., Manica A., Edwards J.S. & Bhak J. (2014) Whole genome sequence and analysis of the Marwari horse breed and its genetic origin. *BMC Genomics* **15**, S4-S.

Khanshour A., Conant E., Juras R. & Cothran E.G. (2013) Microsatellite analysis of genetic diversity and population structure of Arabian horse populations. *J Hered* **104**, 386-98.

Lohmann K. & Klein C. (2014) Next generation sequencing and the future of genetic diagnosis. *Neurotherapeutics* **11**, 699-707.

Makvandi-Nejad S., Hoffman G.E., Allen J.J., Chu E., Gu E., Chandler A.M., Loredo A.I., Bellone R.R., Mezey J.G., Brooks S.a. & Sutter N.B. (2012) Four loci explain 83% of size variation in the horse. *PLoS ONE* **7**, e39929-e.

McCue M.E., Bannasch D.L., Petersen J.L., Gurr J., Bailey E., Binns M.M., Distl O., Guérin G., Hasegawa T., Hill E.W., Leeb T., Lindgren G., Penedo M.C.T., Røed K.H., Ryder O.a., Swinburne J.E., Tozaki T., Valberg S.J., Vaudin M., Lindblad-Toh K., Wade C.M. & Mickelson J.R. (2012) A high density SNP array for the domestic horse and extant Perissodactyla: utility for association mapping, genetic diversity, and phylogeny studies.

PLoS genetics **8**, e1002451-e.

McDonald R.E., Fleming R.I., Beeley J.G., Bovell D.L., Lu J.R., Zhao X., Cooper A. & Kennedy M.W. (2009) Latherin: A Surfactant Protein of Horse Sweat and Saliva. *Plos One* **4**, e5726.

Petersen J.L., Mickelson J.R., Cothran E.G., Andersson L.S., Axelsson J., Bailey E., Bannasch D., Binns M.M., Borges A.S., Brama P., da Câmara Machado A., Distl O., Felicetti M., Fox-Clipsham L., Graves K.T., Guérin G., Haase B., Hasegawa T., Hemmann K., Hill E.W., Leeb T., Lindgren G., Lohi H., Lopes M.S., McGivney B.A., Mikko S., Orr N., Penedo M.C.T., Piercy R.J., Raekallio M., Rieder S., Røed K.H., Silvestrelli M., Swinburne J., Tozaki T., Vaudin M., C M.W. & McCue M.E. (2013) Genetic Diversity in the Modern Horse Illustrated from Genome-Wide SNP Data. In: *Plos One* (

Pirault P., Danvy S., Verrier E. & Leroy G. (2013) Genetic structure and gene flows within horses: a genealogical study at the french population scale. *Plos One* **8**, e61544-e.

Salipante S. J. S.D.J., Rosenthal C, Costa G, Spangler J, Sims, E. H.,Jacobs, M. A., Miller, S. I., Hoogestraat, D. R., Cookson, B. T., McCoy, C. M.,Matsen, F. A., Shendure, J., Lee, C., Harkins, T. T., & Hoffman, N. G (2013) Rapid 16S rRNA Next-Generation Sequencing of Polymicrobial Clinical Samplesfor Diagnosis of Complex Bacterial Infections. *Plos One* **8(5)**.

Towers R.E., Murgiano L., Millar D.S., Glen E., Topf A., Jagannathan V., Drogemuller C., Goodship J.A., Clarke A.J. & Leeb T. (2013) A Nonsense Mutation in the IKBKG Gene in Mares with Incontinentia Pigmenti. *Plos One* **8**.

Yan Y., Yi G., Sun C., Qu L. & Yang N. (2014) Genome-Wide Characterization of Insertion and Deletion Variation in Chicken Using Next Generation Sequencing. *Plos One* **9**, e104652.