

DE NOVO SNP CALLING AND DEMOGRAPHIC INFERENCE USING TRIO
GENOME DATA

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF INFORMATICS OF
THE MIDDLE EAST TECHNICAL UNIVERSITY

BY

ELİF BOZLAK

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE
IN
BIOINFORMATICS

JULY 2019

Approval of the thesis:

DE NOVO SNP CALLING AND DEMOGRAPHIC INFERENCE USING TRIO GENOME DATA

Submitted by ELİF BOZLAK in partial fulfillment of the requirements for the degree of **Master of Science in the Department of Bioinformatics, Middle East Technical University** by,

Prof. Dr. Deniz Zeyrek Bozşahin
Dean, **Graduate School of Informatics, METU**

Assoc. Prof. Dr. Yeşim Aydın Son
Head of Department, **Health Informatics, METU**

Asst. Prof. Dr. Aybar Can Acar
Supervisor, **Health Informatics, METU**

Assoc. Prof. Dr. Mehmet Somel
Co-Supervisor, **Biological Sciences, METU**

Examining Committee Members:

Prof. Dr. Tolga Can
Computer Engineering Dept., METU

Asst. Prof. Dr. Aybar Can Acar
Health Informatics Dept., METU

Assoc. Prof. Dr. Mehmet Somel
Biological Sciences Dept., METU

Assoc. Prof. Dr. Özlen Konu
Molecular Biology and Genetics Dept., Bilkent
University

Assoc. Prof. Dr. Nurcan Tunçbağ
Health Informatics Dept., METU

Date:

29.07.2019



I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Name, Last name : ELİF BOZLAK

Signature : _____

ABSTRACT

DE NOVO SNP CALLING AND DEMOGRAPHIC INFERENCE USING TRIO GENOME DATA

Bozlak, Elif

MSc., Department of Bioinformatics

Supervisor: Asst. Prof. Dr. Aybar Can Acar

Co-Supervisor: Assoc. Prof. Dr. Mehmet Somel

July 2019, 99 pages

De novo mutations are novel mutations which are found in the offspring but not the parents and do not obey the Mendelian inheritance rules. Determining how many de novo mutations occur is important for genetic studies since they help to understand the evolutionary history of populations. In this thesis, we aim to examine de novo mutations that occur within one generation in domestic horses and make estimations on horse demographic history. We used DNA-sequencing data produced by next-generation sequencing technologies from trio data of three different horse breeds: Lipizzaner, Noriker, Haflinger. After quality checks and mapping of the raw data we called genomic variants with three different variant calling algorithms. We filtered all variants depending on their qualities to detect de novo candidates and the final 50 de novo candidates were tested using Sanger resequencing. About 40% of the candidate variants could be validated. We found a higher number of true positives in highly covered Lipizzaner ($n=13$) data, while a lower number of true positives in the low covered Noriker ($n=3$) and Haflinger ($n=5$) data, showing the importance of sequencing coverage to detect true de novo mutations. In addition, we used the Pairwise Sequentially Markovian Coalescent (PSMC) model and performed runs of homozygosity (ROH) analyses to estimate demographic history. Both PSMC and ROH results were coherent with previous studies. All in all, we had an idea for the minimum coverage threshold and quality of whole genome sequencing data, to determine de novo mutations and to estimate population demography.

Keywords: Whole genome sequencing, de novo mutation, Variant calling, Mutation rate, Demography analysis

ÖZ

TRIO VERİSİ İLE DE NOVO SNP ÇAĞIRMA VE DEMOGRAFİK GEÇMİŞ ANALİZİ

Bozlak, Elif

Yüksek Lisans, Biyoenformatik Bölümü

Tez Yöneticisi: Dr. Öğr. Üyesi Aybar Can Acar

Tez Eşyöneticisi: Doç. Dr. Mehmet Somel

Temmuz 2019, 99 sayfa

De novo mutasyonlar ebeveynlerde görülmezken yavruda ortaya çıkan ve Mendel kalıtım kurallarına uymayan mutasyonlardır. Popülasyonların evrimsel tarihlerinin anlaşılmasında yardımcı oldukları için de novo mutasyonların sayılarının tespit edilmesi genetik çalışmalar için önemlidir. Bu tezde evcil atlarda bir jenerasyonda ortaya çıkan de novo mutasyonları tespit etmeyi ve atların demografik tarihleri üzerine tahminler yapmayı hedefledik. Çalışmada üç farklı at türü (Lipizzaner, Noriker ve Haflinger) için yeni nesil sekanslama teknolojisi ile üretilen üçleme DNA sekans verilerini kullandık. Ham verinin kalite kontrolü ve hizalanmasından sonra, üç farklı varyant çağırma algoritması kullanarak genomik varyantları çağırdık. Tüm varyantları kalitelerine göre filtreledik ve seçilen 50 varyantı Sanger sekanslama ile laboratuarda test ettik. Test edilen varyantların yaklaşık olarak %40'ı valide edildi. Yüksek okuma derinliğine sahip Lipizzaner (n=13) türündeki gerçek pozitif sayısını yüksek, düşük okuma derinliğine sahip Noriker (n=3) ve Haflinger (n=5) türlerindeki ise daha düşük sayıda bulduk. Sonuçlar gerçek pozitif de novo mutasyonların tespit edilmesinde okuma derinliğinin önemini gösterdi. Ek olarak elimizdeki at popülasyonlarının demografik tarihleri hakkında tahmin yürütmek için PSMC modeli oluşturduk ve ROH analizi yaptık. PSMC ve ROH sonuçları önceki çalışmalarla uyumlu sonuçlar verdi. Sonuç olarak tüm genom sekanslama verisi ile de novo mutasyon tespiti ve popülasyon demografisi tahmini yapabilmek için gereken minimum veri okuması ve kalitesi hakkında fikir sahibi olduk.

Anahtar Sözcükler: Tüm genom sekanslama, de novo mutasyon, Varyasyon çağırma, Mutasyon oranı, Demografi analizi



To My Family

ACKNOWLEDGMENTS

First of all, I would send my profound thanks to my advisor Asst. Prof. Dr. Aybar Can Acar, my co-advisor Assoc. Prof. Dr. Mehmet Somel and my unofficial co-advisor Dr. Barbara Wallner. They never stop to support me with endless patience and concern during my master's years and they always supported me not just in my academic issues but also in the social issues.

I would also thank my thesis committee members: Prof. Dr. Tolga Can, Assoc. Prof. Dr. Özlen Konu and Assoc. Prof. Dr. Nurcan Tunçbağ, first for accepting to be in my committee and second their precious ideas during the evaluation of my study. In addition to this I owe Tolga Can for the best course that I ever take and his friendly attitudes all the time; I owe Özlen Konu for the positive energy during my thesis period; I owe Nurcan Tunçbağ for her support and trust in many different areas during my master.

I would have special thanks to Dr. Barbara Wallner for providing me the data for my study. She completely trusted and supported me during the analysis of the data. Also, many thanks to Doris Rigler and Eva Michaelis for their time in the laboratory for the validation of the findings.

I would also add my gratitude to Informatics Institute and the 'Music Within' team. Our institute is very supportive and open-minded for new ideas and I saw this attitude at firsthand. My supervisors in 'Music Within' project, Asst. Prof. Dr. Aybar Can Acar and Asst. Prof. Dr. Elif Süreer, never says no to me when I go with the project idea and they always supported me during the project. Besides, Elif Süreer gave me many different perspectives not just for the study and I learned many things from her. A special thanks to Assoc. Prof. Dr. Yeşim Aydın Son and Burcu Üntekin for sharing their data for us for the project. Besides, I would like to thank Yeşim Aydın Son for my great three years in our Bioinformatics Department. I would also thank Ali Çınar for his unrequited help which is an inspiration source to me during the 'Music Within' project. The project was unexpected but very fruitful and fed me in many different aspects during the master.

I also owe special thanks to my family who has an endless support, patience and pure love to me and my ideas all the time: to my brother Barış Bozlak for his pure-minded character, to my mother Nurten Bozlak for her endless support and balancing energy, and my father Metin Bozlak for his strong and promotive personality. There is no doubt that we always try to understand and make the best for each other as shown in my thesis period. Additionally, I believed that they give me a very range perspective on life in many different areas.

I would also send my special thanks to Polat family Suyla Polat, Dilara Polat, Deniz Polat and Haşim Polat for their endless support to me and their endless energy. I can not explain how much I learned from them about life, besides all our music and art sharing. Just luckily, we met again in the Ankara and shared lots of precious moments mostly include brainstormings. Their support during my thesis and also other activities are also nonignorable.

I would have many thanks to my biggest fortunes during my master's years in METU. First, the best colleagues and besties someone can have who are Cansu Demirel, Cansu Dinçer, Evrim Fer, Gökçe Senger, Meriç Kınalı and Muazzez Çelebi Çınar. I cannot tell how I am lucky to know all of them not just in academic cases but also about our sharing on life. Second, my colleagues from the team CompEvo, especially Erinç Yurtman, Reyhan Yaka and Zeliha Gözde Turan. I learned a lot from the team during my master and they supported me all the time. Additionally, I would like to thank my two societies which are METU Capoeira and METU Classical Guitar for many different experiences and the incredible persons they earned to me. We shared many special times and overcome many difficulties with both team and I will never forget the time passed with them. I would send my special thanks to Emre Coşkuner and Burcu Çırtlık for all our special sharing and their endless supports in many different areas during these years. Finally, I would like to thank team MOMO for their promotive attitude and positive vibes during my thesis period.

In the end, I would send special thanks to our department secretary Hakan Güler for his smiling face and patience for us all the time. Because all these people are parts of METU I would send my gratitude to METU and its culture. I can say that, finally I will graduate from a school that I love. I think that METU is one of the precious sides of our country despite it changes with time. I would thank METU for giving me many new perspectives and many precious people.

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LIST OF ABBREVIATIONS

NGS	Next Generation Sequencing
SNP	Single Nucleotide Polymorphism
SNV	Single Nucleotide Variant
PSMC	Pairwise Sequentially Markovian Coalescent
ROH	Runs of Homozygosity
TMRCA	Time to the most recent common ancestor
HMM	Hidden Markov Model
VCF	Variant call file

CHAPTER 1

1.INTRODUCTION

Since horses were domesticated 5000 years ago, they have become a companion to humankind in different areas including transportation, agricultural activities, and wars (Anthony, 2016; Kuderna et al., 2017). Besides these physical advantages, the emotional connection between horses and humans cannot be ignored (Lanata, Guidi, Valenza, Baragli, & Scilingo, 2016). Moreover the domestication of the horses followed by intensive breeding is a very strong example of artificial selection (Zhang et al., 2018), making the evolutionary history of the horse tempting to investigate. Indeed, horses are also chosen as a model organism for the order Perissodactyla (Jagannathan et al., 2018; Wade et al., 2009).

Because of all these reasons above, horses are one of the most well-studied mammals in genetic terms. An updated reference genome of the domestic horse (*Equus caballus*) has recently been released by Kalbfleisch and colleagues (Kalbfleisch et al., 2018). After the release of the updated reference, Jagannathan et al. created a comprehensive variation panel for the modern domestic horse, *Equus caballus* (Jagannathan et al., 2018). In this thesis, we will be analyzing genomic data from modern horses using these resources.

The subject of the thesis will be genetics of three well studied modern horse breeds, namely the Lipizzaner, Noriker and Haflinger. What is common for these horses is that, they all are Austrian breeds. Lipizzaner horses are bred, among other studs, in the Piber stud in Austria and they are selected for dressage performance in the Spanish Riding School (Grilz-Seger et al., 2019). The Noriker is an Austrian draft horse breed and bred for agricultural purposes in the Alps (Druml et al., 2018). And the last breed, which is known as the youngest horse of Austria, is the Haflinger (Druml et al., 2018).

The thesis focuses on the identification of germline ‘de novo’ mutations. ‘De novo’ mutations represent novel mutations occurring in one generation. They are one of the driving sources of gaining new variants throughout generations (Campbell & Eichler, 2013) and they are one of the main contributors of several genetic diseases (Jin et al., 2017). ‘De novo’ mutations are studied from several different aspects in population genetics, as mutation rate per generation is important to study evolutionary history of

populations (Smeds, Qvarnström, & Ellegren, 2016; Tatsumoto et al., 2017). Hence bioinformaticians have spent intense effort on developing ‘de novo’ mutation detection pipelines and algorithms (Acuna-Hidalgo, Veltman, & Hoischen, 2016; Francioli et al., 2017).

There are two main ways of calculating the ‘de novo’ mutation rate, either using phylogenetic distance methods or directly detecting ‘de novo’ mutation candidates in one generation (Campbell & Eichler, 2013). For the well-studied genomes such as that of human, the mutation rate has been calculated via both methods. This provides accuracy in population and medical genetics analyses that depend on the mutation rate. For horses, the only estimate yet has been made by Orlando et al., who calculated the mutation rate for the domestic horse with the phylogenetic distance method. The authors used comparative genomics data from different *Equus caballus* genomes and a donkey genome to estimate the mutation rate (Orlando et al., 2013). Although this rate was used in different horse studies later, there is no mutation rate estimated using ‘de novo’ mutations for the horse. Providing such an estimate is my main motivation in this thesis.

One application of such rate estimates is in evolutionary demography. Population demography analysis attempts to understand past and present population dynamics, such as bottlenecks, migrations or inbreeding levels (Nielsen & Slatkin, 2013). One way of doing this is using genomic data, and this approach is frequently used to make estimation for the structures of the populations in the past. Pairwise Sequentially Markovian Coalescent (PSMC) is one of these methods to make estimations about population history from genomic data. The algorithm makes estimates of the effective population size at different time points from a single diploid genome (Li & Durbin, 2011). PSMC analysis has been recently used to study horse demographic history as well. Different PSMC studies on horse genomes gave consistent results with each other (Orlando et al., 2013; Schubert et al., 2014). More specifically, these found a decrease in the horse effective population size in the Last Interglacial Period followed by a peak after the Last Glacial Period. The mutation rate used in these studies was again that estimated by Orlando and colleagues (Orlando et al., 2013; Schubert et al., 2014).

Another method used to estimate demographic history is runs of homozygosity (ROH). The results of ROH analysis allow inferences on the amount of inbreeding and presence of bottlenecks in a population in the recent past (Ceballos, Joshi, Clark, Ramsay, & Wilson, 2018; Kirin et al., 2010). Previous works have studied the three horse populations in this study (Austrian Lipizzaner, Noriker and Haflinger) using both pedigree and ROH analyses, and found higher inbreeding in Lipizzaner and Haflinger than in Noriker horses (Druml, Baumung, & Sölkner, 2009; Grilz-Seger et al., 2019, 2018).

In this thesis we analyzed NGS data of trios from the three different domestic horse populations mentioned above. We used data collected from trios, consisting of a mother, a father and an offspring from each of these three breeds. The data were produced by the research group of Dr. Barbara Wallner, Institute of Animal Breeding and Genetics, University of Veterinary Medicine Vienna. The main aim of this study was to detect ‘de

novo' mutations that are found only in the offspring of these breeds. 'De novo' mutations were predicted and mutation rates per generation were inferred for the domestic horse. This study is thus the first to calculate horse mutation rate per generation, inferred from genome-wide 'de novo' mutations. We further used the data to make demographic estimations for demography of these three populations using PSMC and ROH analyses, and the results were compared with those of the previous studies.

The second chapter of the thesis describes the importance of 'de novo' mutations in the fields of genetics and bioinformatics, as well as giving examples of mutation rate estimates and their calculation methodologies. Besides, we compare the different bioinformatics algorithms that we used for the analyses, especially for variant calling. Finally, we mention working strategies of PSMC and ROH methods and findings from different studies for domestic horse populations.

The third chapter explains our methodology to analyze whole-genome NGS data in detail to detect 'de novo' mutations produced in one generation. Here we used different calling algorithms and two different reference genomes. The chapter also details how we performed PSMC and ROH analysis.

The fourth and fifth chapters describe our results and compare them with previous studies. Then, we make inferences on stability of our results and discuss possible limitations and future directions.



CHAPTER 2

2.LITERATURE REVIEW

2.1. Mutation rates and 'de novo' mutations in evolutionary studies

Germline mutations are one of the fundamental forces of evolutionary mechanisms due to their contribution to the variation pool of populations (Loewe & Hill, 2010). Besides, they are also one of the causes of heritable and complex diseases (Shendure & Akey, 2015). These properties necessitate making accurate predictions on mutation rates (Narasimhan et al., 2017; Shendure & Akey, 2015). In addition to some recent population genetic methods, there are two main ways of estimating mutation rates. The first one is calculating mutation rates based on phylogenetic distances. In this method, comparative genomic data from two lineages with known splitting time (based on the fossil record) is used to calculate the nucleotide divergence between the two. This divergence can then be used to estimate the per generation substitution rate, which can also be used as the per generation mutation rate. This approach is based on the neutral theory of molecular evolution, which states that for neutral loci, the substitution rate should be equal to the mutation rate (Nielsen & Slatkin, 2013). Furthermore, it assumes that the generation time is known and fixed for the lineages in question. The other method for calculating the mutation rate is using 'de novo' mutations. In this approach, direct calculations from genomic data of a trio (including mother, father and offspring data) is used and the number of mutations only observed in the offspring is divided by the genome length (Campbell & Eichler, 2013; Narasimhan et al., 2017; Shendure & Akey, 2015).

There are several studies for mammals which have used the phylogenetic distance method for mutation rate calculations. For instance, the mutation rate was estimated as $\sim 2 \times 10^{-8}$ per base per generation for human and nonhuman primates by considering divergence between neutral sites in their genomes following the above approach (Mikkelsen et al., 2005; Shendure & Akey, 2015). The rate has been reestimated as 1×10^{-9} per base per year with the same method in different studies (Prüfer et al., 2012) by assuming the divergence time between human and chimpanzee as 6-7 million years ago (Ma). For the domestic

horse, Orlando et al. estimated the mutation rate per generation with the phylogenetic distance method (Orlando et al., 2013). They estimated the mutation rate as $7.2e-09$ per site per generation by comparing horse and donkey, and assuming that donkey and horse diverged 4-4.5 Ma (Janečka et al., 2018; Jónsson et al., 2014). Both primate and equid phylogenetic mutation rate estimates thus appear roughly within the same order of magnitude (the estimate for equids being midway between different estimates for primates).

Although the phylogenetic distance method has been frequently used in many different studies to estimate the mutation rate, scientists have pointed out multiple weaknesses of this method. Tatsumoto et al. suggested that different factors such as generation time, effective population size, rate of heterogeneity causes uncertainty in results when using the phylogenetic approach (Tatsumoto et al., 2017). Francioli and his team also argued for the disadvantages of the method because different selection mechanisms are effective on different populations and species, and across different regions of the genome, and therefore identifying truly neutral regions will not be straightforward (Francioli et al., 2014).

Given these problems of the mutation rate estimation based on phylogenetic distance, and thanks to the availability of high throughput (next generation) sequencing technologies, scientists in recent years have started making direct estimates of the mutation rate by 'de novo' mutations scans. These studies use high quality whole genome data from trios (for sexually reproducing species) produced by next generation sequencing. To date, mutation rate estimations from 'de novo' mutations have been made in a few mammalian species, such as humans, chimpanzee and mouse. While some studies compared different families of the same population (Francioli et al., 2014; Jónsson et al., 2017), others focused on families from different populations (Conrad et al., 2011). For example, Jonsson and colleagues worked with a large genomic data dataset comprising 1,548 human trios from Iceland. They made estimations on the 'de novo' mutation rate in one generation and they estimated the rate as $1.28e-08$ per base per generation. Meanwhile, Francioli and colleagues analyzed genomic data from 250 Dutch parent-offspring families including trios and twin-parent families, and they calculated mutation rates for different parts of the human genome to understand possible explanations for the different mutation rates. Conrad and colleagues calculated and compared mutation rates inferred from trios of European descent ($u=1.17e-08$ bp/gen) and Yoruban descent ($u=0.97e-08$ bp/gen) and found possible sources for the differences between two populations. In addition to human studies, nowadays 'de novo' mutation studies are also being conducted in other mammalian species. Tatsumoto et al. produced ultra-deep sequencing data for a chimpanzee trio to calculate 'de novo' mutation rate for the chimpanzees, and they estimated this as $1.48e-08$ per site per generation (Tatsumoto et al., 2017). Another popular mammal subjecting for mutation rate calculations is mice (*Mus musculus*). Uchimura and colleagues calculated the germline mutation rate for mice is $5.4e-09$ bp/gen (Uchimura et al., 2015) and Lindsay and colleagues calculated the rate as $3.9e-09$ (Lindsay, Rahbari, Kaplanis, Keane, & Hurles, 2016). Both rates were calculated using the number of 'de novo' mutations detected in the offspring but not in the mouse population of interest. Intriguingly, the

mutation rate of humans appeared almost 2 to 3 times higher than the mouse. This difference has been explained, by Lindsay and colleagues, by the larger effective population size of the mouse (and thus stronger selection for high fidelity DNA copying) when compared with the human (Lindsay et al., 2016). Notably, the resulting rates from these studies were comparable with each other irrespective of populations and species, and also consistent with the rates that were calculated with the phylogenetic distance methods for human, all being on the order of $\sim 1e-08$ bp/gen.

Narasimhan and colleagues have discussed possible reasons for the discrepancies between mutation rates estimated with the two different methods (phylogenetic distance vs. direct estimations from ‘de novo’ mutations). They suggested different explanations such as i) sample size (including number of individuals used in the study or number of true ‘de novo’ mutations); ii) using only mutations seen in one generation; iii) effect of paternal age; iv) genomes of diseased individuals (Narasimhan et al., 2017). Additionally, Conrad et al. showed that, although mutation rates were calculated with the same methods in different trios of same species, differences in these rates could also be observed (Conrad et al., 2011). They suggested different reasons for this difference. The first one is variation due to differences in genetic background and selection dynamics among individual. For instance, although it is accepted that more mutations are occurring in the paternal lineages (Haldane, 1947), Conrad et al. found a different pattern in one of their studied families. In this family a higher percentage of ‘de novo’ mutations derived from the maternal lineage. The second possible reason is the age of parents. This idea is also supported by different studies. Kong and team showed that the increasing age of the father results in an increase in the number of ‘de novo’ mutations occurring in one generation (Kong et al., 2012), a conclusion also supported by Francioli et al. (Francioli et al., 2014). In addition to these reasons, Francioli et al. found that different genomic regions could have different mutations rates. This can create a bias in mutation rate estimates from whole genome data depending on the distribution of number of reads mapped along the genome.

2.2. Importance of data amount and quality in next-generation sequencing analysis

2.2.1. Quality of whole-genome data

Different studies show the importance of sequencing depth in detecting true ‘de novo’ mutations. Tatsumoto and team estimated mutation rate with their ultra-deep high-quality sequencing data (Tatsumoto et al., 2017). To do this, they validated their candidate ‘de novo’ SNPs by Sanger sequencing. They categorized their data as 30x, 60x, 90x and 120x groups, dependent on the sequencing depth. Then they took the 90x and 120x candidate lists and found all true positive mutations ($n=32$) in the shared list of the two categories. On the other hand, there were 9 candidates found only in 90x data, and these were all detected as false positives in Sanger analysis. Based on these results, they suggested that even 90x coverage is not enough to eliminate all false positives. According to this study, determining true positive ‘de novo’ SNPs, which includes eliminating false positives and

avoiding false negatives, can only be revealed with ultra-deep covered data (Tatsumoto et al., 2017). 'De novo' mutation studies in human trios have also supported these results. Jonsson et al. used data with an average of 35x coverage and Conrad et al. used data greater than 22x coverage, and their mutation rate estimates per generation ($1.28e-08$ in Jonsson et al. and $\sim 1.2e-08$ in Conrad et al.) were consistent with each other (Conrad et al., 2011; Jónsson et al., 2017). Although, Tatsumoto et al. suggested that these coverages are not sufficient to detect 'de novo' mutations, Jonsson detected on average 70.3 'de novo' mutations in 1548 families trio data and Conrad detected 49 and 35 'de novo' mutations in the two trios they worked with. This is an indication of uncertainty about the lower data thresholds to detect 'de novo' mutations.

2.2.2. *Quality of reference genome*

The quality of the reference genome is another factor that affects mapping rates, variant calls and consequently 'de novo' mutation rate estimates (Kalbfleisch et al., 2018; Li & Wren, 2014). An increase in the mapping rates was seen in the updated human reference genome from GRCh38 when compared with GRCh37 (Guo et al., 2017). Besides this increase in the mapping, Guo et al. also detected fewer number of SNVs in the GRCh38. They explained the reason for this lower number of variants using GRCh38 by fewer false positive variants due to quality of new reference. In addition, Li and Wren compared human references GRCh37 and GRCh38 in their analysis and showed the effects of different parameters in variant calling (Li & Wren, 2014). They also found fewer false positive variants when using the updated reference.

In the case of horse, the first high-quality reference assembly for the domestic horse, EquCab2, was released by Wade et al. (Wade et al., 2009). The updated version of the *Equus caballus* reference genome was recently published in 2018. The data which were used to construct the updated reference was derived from the same Biosample, the female Thoroughbred 'Twilight'. In the updated version of the reference genome EquCab3 different sequencing and assembly technologies were used. In the updated reference genome, the number of non-N bases on the chromosomes were increased to 2.41 Giga base (Gb) from 2.33 Gb in EquCab2. Besides, the gaps in the incorporated chromosomes decreased 10-fold in the EquCab3. To test the updated reference, whole genome sequencing data from two Thoroughbred male horses were mapped to both EquCab2 and EquCab3 references (Kalbfleisch et al., 2018). These authors found an increased number of mapped reads and properly paired mapped reads on the EquCab3 mapped data when compared to that of EquCab2. Kalbfleisch et al. suggested that this might be a result of the improvement in the updated genome in several ways, such as containing fewer gaps (Kalbfleisch et al., 2018). All in all, these comparative studies suggest that an updated reference genome that includes deep coverage high throughput sequencing data, may provide more accurate results.

2.2.3. *Quality and properties of existing variation panels*

The importance of variation panels in the ‘de novo’ mutation detection workflows was previously mentioned by Romero and colleagues (Gómez-Romero et al., 2018). These authors suggested that, because there is a low probability for the occurrence of independent ‘de novo’ mutations at the same position in different individuals, standing variation can be used to eliminate false positives, which leads then to more accurate ‘de novo’ mutation estimates. They filtered their candidates in the study, based on this idea. In the recent past, 50k, 70k and 670k SNV variation panels were produced for targeted genotyping arrays with previous reference genomes of the domestic horse (McCoy & McCue, 2014; McCue et al., 2012; Schaefer et al., 2017). A variation panel produced from whole genome sequencing data of 88 horses from different breeds was recently released (Jagannathan et al., 2018). In this panel the updated version of the reference (EquCab3) was used as reference genome. Jagannathan et al. called approximately 23.5 million SNVs in this study. This newly constructed horse variation panel based on EquCab3 by Jagannathan et al. can also be used as an efficient tool to eliminate false positives to detect true ‘de novo’ candidates in horse trios.

2.3. Analyzing high throughput sequencing data for ‘de novo’ mutation detection

2.3.1. *Differences among variant calling algorithms*

There are several tools developed to find variations from the reference genome and variations among different individuals. In recent years multiple studies have compared the performances of different variant callers. Poplin et al. has recently reviewed different variant calling algorithms and compared their performances (Poplin et al., 2017). These algorithms can be split into two main groups. One group finds mismatches between the subject individual and the reference genome. These are called ‘pileup’ callers, and SAMtools (Li et al., 2009) and GATK’s UnifiedGenotyper (UG) (Depristo et al., 2011) are examples of this group. Despite their high sensitivity, these algorithms have some weak points caused by using a reference sequence to find variants (Rimmer et al., 2014) or considering one position at a time during variant calling process (Garrison & Marth, 2012; Rimmer et al., 2014). The other group of variant caller algorithms are called assembly-based algorithms; these basically construct haplotypes by creating de Bruijn-like graphs. Platypus (Rimmer et al., 2014), GATK’s HaplotypeCaller (HC) (Poplin et al., 2017) and FreeBayes (Garrison & Marth, 2012) are examples of this group of variant callers (Poplin et al., 2017; Xu, 2018). These tools first assemble reads locally and produce candidate haplotypes; then they estimate likelihood of haplotypes by aligning the reads to the haplotypes one by one and counting them. The advantages of these latter algorithms becomes most obvious in highly variable genomic regions, because they do not depend on local alignment as ‘pileup callers’ do, which causes mistakes in this high variable regions (Li & Wren, 2014; Poplin et al., 2017; Rimmer et al., 2014). In addition, assembly-based algorithms can detect the co-existence of different variants at the same time (Xu, 2018). Poplin and team compared variant callers including both types of algorithms

(‘pileup’ and assembly-based) and among four algorithms, namely GATK’s HaplotypeCaller (HC), GATK’s UnifiedGenotyper (UG), Platypus, SAMtools, they found the highest sensitivity in HC. The authors also suggested that assembly-based algorithms give more accurate results. Another idea that is also proposed in the HaplotypeCaller’s paper is that calling variants jointly in multiple individuals improves accuracy of the calling results, especially for the regions with low coverage (Poplin et al., 2017; Rimmer et al., 2014), but such joint calling results in the production of more complex de Bruijn graphs, so it needs more computational power (Poplin et al., 2017). HaplotypeCaller, Platypus and FreeBayes try to solve this computational complexity in the algorithms while they make haplotype-based variant calling.

Sandmann and colleagues used and compared GATK’s HC, Platypus and FreeBayes besides other variant callers on their targeted Illumina HiSeq data (Sandmann et al., 2017). The main aim of their study was to produce a fast pipeline to use in clinical applications to identify both rare and common variants. After analyzing their data with different variant calling algorithms, they reported that Freebayes is the most sensitive among these three variant callers, despite reporting many false positive variants. They further reported that the accuracy of Platypus’s results depends on allele frequency of the variant and that of HaplotypeCaller depends on the type of platform, specifically whether HiSeq or NextSeq was used to produce the data (Sandmann et al., 2017).

Some algorithms like PhaseByTransmission (PBT) (Francioli et al., 2017) are designed to directly use known pedigree information of trio data and possibly allele frequency in the population, which can be further used to detect ‘de novo’ mutations. PBT calculates posterior probabilities of each candidate variant and outputs a list of candidates (Francioli et al., 2017). Francioli and team compared their PBT algorithm with other ‘de novo’ detection tools, namely DeNovoGear (Ramu et al., 2013) and TrioDeNovo (Wei et al., 2015). They found consistent results using high coverage data, but they found PBT results to be more accurate using relatively low coverage data downsampled to 15x. On the other hand, PBT found many false positive ‘de novo’ candidates which could not be validated in the low coverage data (2-20x) in all trio members.

2.3.2. *Importance of filtering variants*

Filtering variant lists produced by calling algorithms is a mandatory step to eliminate false positive variants as much as possible. Li and Wren made a comprehensive analysis to show how the filtering steps affects the quality of analysis (Li & Wren, 2014). They used FreeBayes, GATK UnifiedGenotyper, Platypus and GATK HaplotypeCaller for variant calling in their analysis and they filtered their data depending on different parameters: low-complexity regions, maximum depth, allele balance, double strand filter, Fisher strand filter, quality filter. The authors found that although the low complexity region filter is the most effective filter to eliminate false positive heterozygous variants overall, when using data aligned with the BWA-mem, the maximum depth filter is the most effective filter among other filters. The other parameters gave different result for the different call sets (Li & Wren, 2014). One of the other well-accepted filtering strategies is GATK’s hard

filtering strategy which is suggested by Summa and colleagues (De Summa et al., 2017). These authors tried to identify parameters for 7 standard GATK filters followed by a classification tree to decide correct variants.

Besides the general filters mentioned above, there are several other strategies to detect ‘de novo’ mutations using genomic variation data. Jonsson et al. used minimum depth, allele balance and genotype likelihood parameters in their works to define their ‘de novo’ mutation candidates (Jónsson et al., 2017). Tatsumoto et al. also used read depth and allele balance in their ‘de novo’ SNV defining strategy (Tatsumoto et al., 2017). Li and Wren mentioned the importance of depth and allele balance in filtering (Li & Wren, 2014). While positions having low depth (depending on the study) are not significant, positions with high depth are not efficient for the detection of true ‘de novos’ (Jónsson et al., 2017; Li & Wren, 2014; Tatsumoto et al., 2017). Allele balance filter is also meaningful to eliminate mutations which show low proportions of alternative allele to reference allele counts, which is indicative of technical errors (Li & Wren, 2014).

2.4. Demography analysis in *Equus caballus*

Different estimations on demography such as population size, migration, inbreeding or history of divergence could be made with population genetics data (Nielsen & Slatkin, 2013). With the increase in the amount and abundance of genomic data, new methods are being developed to make estimations on the demographic history of populations. Estimating evolutionary trees or making cluster analysis between individuals of a population could be some examples for demography analysis. Here we used two population genetics methods, pairwise sequentially Markovian coalescent (PSMC) and runs of homozygosity (ROH), to study horse demographic history.

2.4.1. Pairwise Sequentially Markovian Coalescent (PSMC)

PSMC is a haplotype-based method to make estimations on population demography from genomic information (Chen, 2015; Li & Durbin, 2011). The main idea behind the method is that, using whole genome diploid sequence data from a single individual, changes in past effective population sizes can be estimated. It uses the proportion of heterozygote sites in a genomic segment separated from other segments by ancestral recombination events and infers the local time to the most recent common ancestor (TMRCA) by using a Hidden Markov Model (HMM). In this HMM model the observation is the diploid sequence of an individual and the hidden states are the TMRCA and ancestral recombination events, the latter represented by transitions. The parameters that are necessary for the calculations are mutation rate, recombination rate and generation time in the population, which are estimated from the data using an expectation-maximization algorithm. Results of the analysis of human whole genome data provides estimates of the changes in the population size between 20 kya to 3 Mya. Getting information for a larger time period is also possible but Li and Durbin suggested that it will not be giving accurate results (Li & Durbin, 2011).

There are different studies which investigated horse demography by the PSMC method. Orlando and colleagues studied genomes of an ancient horse, five modern domestic horses, a Przewalski's horse and a donkey with PSMC analysis. First, they calculated a mutation rate for the horse genome as $7.2e-09$ per site per generation by aligning genome sequences of the horse and the donkey. They assumed 8 years generation time for the horses. Then they performed PSMC analysis using the calculated mutation rate and generation time. They made estimations for the last 2My of the horse population (Orlando et al., 2013). Their results suggested that the horse population size reached a minimum around 125 kya before present, a time period that coincides with the Last Interglacial Period. On the other hand, the population size reached the maximum level between 25-50 kya, which corresponds to climatic changes fruitful for the vegetation after the Last Glacial Maximum (Lorenzen et al., 2011). Schubert and team also made PSMC analysis for the horses by using parameters from Orlando et al.'s 2013 study. They analyzed another two ancient horse genomes, again together with domestic and Przewalski's horse genomes, to detect signals of domestication by using whole genomes of the horses and found patterns similar to the findings of Orlando and colleagues' study (Schubert et al., 2014).

Although the PSMC analysis gives highly accurate estimations, Orlando et al. also detected a bias in the result for low coverage data (<20x) (Orlando et al., 2013). Nadachowska-Brzyska and colleagues also mentioned the impact of data coverage in the PSMC analysis. They analyzed 200 individuals from four different flycatcher species and made PSMC analysis to have idea of demographic history of their populations. They found that their results are much more consistent using data from individuals having at least 18x mean coverage and applying a 10x coverage filter per-site. They suggested to perform PSMC analysis using this approach (Nadachowska-Brzyska, Burri, Smeds, & Ellegren, 2016).

2.4.2. *Runs of homozygosity (ROH)*

Calculating runs of homozygosity is another technique to detect bottlenecks and inbreeding rate in populations. The idea underlying this technique comes from the distribution of autozygous sites in genomes. Broman and Weber proposed the idea that these sites are not evenly distributed in the genome, but they are dispersed in the form of runs, or tracks (Broman & Weber, 1999; McQuillan et al., 2008). Ceballos and team recently discussed the relationship between ROH sites and population dynamics in a review (Ceballos et al., 2018). First, they showed that the length and distribution of ROHs in a genome are related with population size. The relationship occurs such that smaller populations have longer and more ROHs than larger populations. Then the authors explained how different processes affect the distribution of ROHs. Admixture events cause decrease in the length and number of ROHs, whereas bottlenecks lead to an increase in the number of ROHs but usually keep the length relatively short. Inbreeding is shown to result in an increase in the number of very long ROHs. If a population both went through a bottleneck and exercised inbreeding, the individuals will show both many short ROHs and some very long ROHs (Ceballos et al., 2018).

The horse (*Equus caballus*) is well studied for ROHs by many different groups. Grilz-Seger et al. made ROH analysis with four different sub-populations of Lipizzaner horses (Grilz-Seger et al., 2019). One of the conclusions the authors reached is Austrian Lipizzaner being more inbred than other Lipizzaner populations. They showed that cumulative ROHs (F_{ROH}) in different lengths was almost twice in the Austrian Lipizzaners compared to Croatian, Hungarian, and Slovakian Lipizzaners. In addition, the number of long ROHs (>4Mb) were two to ten times higher in Austrian compared to the other Lipizzaners. Based on these results they suggested Austrian Lipizzaners are more inbred and bottlenecked (Grilz-Seger et al., 2019). Furthermore, they estimated inbreeding coefficients from ROH data, and these estimates were also consistent with the inbreeding coefficients calculated by pedigree analysis for the Austrian Lipizzaner (Zechner et al., 2002). Grilz-Seger therefore suggested that the Austrian Lipizzaner is the most inbred sub-population among Lipizzaners. In addition to the ROH analysis, Druml et al. analyzed pedigree data of Austrian Noriker horses and they suggested that inbreeding in Austrian Noriker is lower than Austrian Lipizzaner (Druml et al., 2009). However, the inbreeding coefficient calculated for the Austrian Haflinger, which was calculated by Druml and team in 2018, was much higher and similar to the one observed in the Austrian Lipizzaner (Druml et al., 2018; Grilz-Seger et al., 2019).



CHAPTER 3

3.MATERIALS AND METHODS

Here, we explain the methodologies using in this study, which covers whole genome sequencing data analysis including read mapping and variant calling, as well as demography analysis.

3.1. Next Generation Sequencing Data

Whole-genome Illumina Next Generation Sequencing (NGS) data for three family trios (in total nine horses) from different breeds were provided by Dr. Barbara Wallner, Institute of Animal Breeding and Genetics, University of Veterinary Medicine in Vienna, Austria. All trios include mother, father and a male offspring. Trio 1 were three purebred Lipizzan horses, trio 2 purebred Noriker horses and trio 3 purebred Haflinger horses. Whole blood samples of each individual were collected for NGS data generation in 2012.

NGS data was generated in different years and on different platforms. The Lipizzaner trio was sequenced by HiSeq2000 at BGI (Beijing Genomics Institute) in 2013, whereas the Haflinger and Noriker data were produced by HiSeqV4 at the Core Facility for Sequencing at the Vienna BioCenter (CSF) in 2015. The data consist of paired-end reads. Read length in Lipizzaner reads was 90 base pairs (bp), whereas the read length in Noriker and Haflinger reads was 125 bp. General information about the data is given in Table 3.1 below.

Haflinger and Noriker data were provided as unmapped BAM files, while Lipizzaner data in FASTQ format. Before starting the quality analysis of raw files, all BAM files were sorted with SAMtools (Li et al., 2009) (see Appendix A for version and command line usage of the tool) and converted to paired-end FASTQ files by BEDTools' (Quinlan & Hall, 2010) (see Appendix A) 'bamtofastq' algorithm.

Table 3.1: General information of individuals and provided data formats of each individual. Some of the individuals were sequenced on multiple lanes.

Sample ID	Breed	Pedigree	Number of sequencing lanes	Initial file format
111	Lipizzaner	Son	3	FASTQ
113	Lipizzaner	Father	3	FASTQ
166	Lipizzaner	Mother	4	FASTQ
BW-352	Noriker	Father	2	BAM
BW-353	Noriker	Mother	1	BAM
BW-354	Noriker	Son	1	BAM
BW-355	Haflinger	Father	1	BAM
BW-356	Haflinger	Mother	2	BAM
BW-357	Haflinger	Son	1	BAM

3.2. First Data Analysis

3.2.1. Quality control and trimming of FASTQ files

Quality controls of the FASTQ files from each sample were performed with FastQC (Andrews, 2015) (see Appendix A). Then, all reads were quality-trimmed with Trimmomatic (Bolger, Lohse, & Usadel, 2014) (see Appendix A) using the following parameters: “TRAILING:10, MINLEN:50, SLIDINGWINDOW:5:20”. These parameters were chosen due to the low-quality reads, some of which were under Phred score of 30 towards the end of the reads. Next, the quality of all trimmed FASTQ files was reinvestigated using FastQC. To visualize FastQC results, the MultiQC software (Ewels, Magnusson, Lundin, & Källner, 2016) (see Appendix A), which creates a report by summarizing FastQC results, was used.

3.2.2. Mapping reads to the reference genomes

First, reference genomes EquCab2 (Wade et al, 2009) and EquCab3 (Kalbfleish et al., 2018) were indexed with BWA (Li & Durbin, 2010) using the ‘bwtsw’ algorithm. Then the ‘mem’ (Li, 2013) algorithm of the BWA, which shows high performance in mapping of long reads was used for mapping reads to the reference genomes. As for some

individuals the FASTQ data was derived from different sequencing lanes (see Table 3.1), data from each lane were mapped to the references separately. SAM files were obtained at the end of the mapping, which were converted to binary BAM files with the SAMtools' 'view' algorithm and these BAM files were sorted with the SAMtools' 'sort' algorithm. For individuals with more than one lane sequenced, each lane was treated in the same way and then data from different lanes were merged with the SAMtools' 'merge' algorithm to create a merged BAM file, which contains data from separate lanes. These BAM files were sorted again, and only properly paired reads were chosen with the SAMtools' 'view' algorithm using the parameters: "-F 4 -f 2 -h". Duplicate reads were removed by the MarkDuplicates algorithm of Picard Tools with the "REMOVE_DUPLICATES=TRUE" parameter. At the last step, reads that were under quality score 20 were filtered out with SAMtools' 'view' again, with the '-q 20' parameter. Insert sizes of the produced BAM files that were mapped to the EquCab3 were calculated by Picard Tools's CollectInsertSizeMetrics function. Finalized BAM files were indexed with SAMtools. Versions of all tools and their command line usages were given in Appendix Table A.A.1 and A.A.2.

3.2.3. *Calculating mapping coverage*

Coverages of the mapped BAM files were calculated with the 'genomecov' function of BEDtools (see Appendix A) for each horse. Then, the mean mapping on autosomal chromosomes for each individual and also for each trio were calculated in R (see Table A.A.1 for version).

To identify positions within a certain depth range, positional coverages of each individual were also calculated using the same function of BEDtools with the "-d" parameter (see Appendix A). The resulting coverage files were separated into chromosomes by UNIX command-line functions. Then, an R code that checked whether a certain position was within the given depth range in each member of the respective trio was written. The thresholds were set to 10 to 30 reads for EquCab2 mapped data, and 10 to two times mean coverage of the respective trio in the EquCab3 mapped data (Li & Wren, 2014).

3.3. **Variant calling**

Three different algorithms were used to call variants including single nucleotide variants (SNV) and insertion-deletion variants (INDEL) in both references. These were i) GATK's 'HaplotypeCaller' (Poplin et al., 2017) function, ii) Platypus' (Rimmer et al., 2014) 'callVariants' function, and iii) the Freebayes (Garrison & Marth, 2012) algorithm (see Appendix A for version and command line usage of the tool). Default parameters of each callers were used, and members of the same trio were given to the callers at once while performing variant calling. The output variant calling file (VCF) of each algorithm included all variants detected in the trio horses when compared to the reference genome. At the end of variant calling step, six different VCF files had been generated per trio, one from GATK, one from Platypus and one from Freebayes, for each reference.

3.3.1. Workflow for determining candidate ‘de novo’ mutations

In the next step, different filters were applied to detect ‘de novo’ mutations, which we define as occurring in the offspring and not found in the parents. To predict ‘de novo’ mutation candidates, a custom Python (see Appendix Table A.A.1 for the version) code was written. The code parsed each variant in the VCF file line by line. First, it checked the variant for any undetermined genotype among the three individuals. Such variants were filtered out. In next step, the code compared the offspring’s genotype with its parents’ genotype. Table 3.2 shows all of the conditions that were chosen as a ‘de novo’ candidate in the offspring. The first four rows of the table show the homozygous cases and last two rows show the heterozygous cases in the offspring.

Table 3.2: ‘de novo’ candidate patterns extracted from VCF files.

Offspring	Parent 1	Parent 2
0/0	1/0	1/1
0/0	1/1	1/0
1/1	0/0	1/0
1/1	1/0	0/0
0/1	0/0	0/0
0/1	1/1	1/1

After choosing ‘de novo’ candidates, chrX, chrUn, chrM, and INDELs (insertions and deletions) were removed by another Python code. At the end, the output files contained SNV ‘de novo’ candidates which are located on the autosomal chromosomes. For the final ‘de novo’ list for each trio, ‘de novo’ SNP candidates called by three different variant calling algorithms were considered. For this we generated BED files from the VCF files using Bedtools (see Appendix A) and then the ‘intersect’ function of Bedtools to choose shared variants. These final position files were converted into VCF files with a Python script. While creating VCF files from BED files, genotype information for VCF files was derived from the GATK variant file of each related trio.

For the next filtering steps, the GATK tool was used. First, the allele balance of each variant was calculated by the ‘VariantAnnotator’ function, with the ‘-A AlleleBalanceBySample’ parameter. Then GATK hard filtering (De Summa et al., 2017) was applied to the VCF data using ‘VariantFiltration’ function with the given parameters: “QD < 2.0, MQ < 40.0, FS > 60.0, SOR > 3.0, MQRankSum < -12.5, ReadPosRankSum < -8.0, QUAL < 30, AC > 4”. ‘de novo’ candidates that passed filtering were kept and heterozygous candidates in each offspring were selected among these with the ‘.isHet()’

function. The last two columns of Table 3.2 show the possible allele distribution of selected variants among trio individuals. In the last step, the genotype statistics (including GT, AD, DP, GQ and PL) of individuals in each trio were filtered with ‘VariantFiltration’ and ‘--genotypeFilterExpression’ functions. Filtering parameters for genotypes were: $AB < 0.3 \parallel AB > 0.75$, $GQ < 40.0$ (Jónsson et al., 2017; Maretty et al., 2017). For the depth (DP) filtering, we required depth between 10x and 30x for the EquCab2 mapped data. In EquCab3 mapped data, we changed the threshold and we required lower and upper thresholds between 10x and 2 times of breed mean coverage, respectively (Li & Wren, 2014). Analysis steps are shown in Figure 3.1 as four main parts (Mapping, Variant Calling, Filtering, Comparison). Versions of tools and their command line usages are given in Appendix Table A.A.1 and A.A.2.

3.3.2. *Determining candidate ‘de novo’ mutations with GATK PhaseByTransmission (PBT) algorithm*

To test the results of the workflow in section 3.3.1, GATK’s PhaseByTransmission (Francioli et al., 2017) algorithm, which predicts candidate ‘de novo’ mutations, was implemented as an alternative strategy. The function was run with the Lipizzaner variants from three different algorithms (GATK HaplotypeCaller, Platypus and Freebayes) separately. The ‘--MendelianViolationsFile’ parameter was added to obtain candidate ‘de novo’ mutations as a list. Shared ‘de novo’ candidates from the three different calling algorithms were selected. GATK’s PhaseByTransmission (see Appendix A) algorithm was applied only for the Lipizzan trio and in the next step (3.3.3) the resulting list of ‘de novo’ candidates was compared to the ones’ predicted with the custom approach described in 3.3.1 (workflow for determining candidate ‘de novo’ mutations).

3.3.3. *Comparing different variant lists*

In the next step, ‘de novo’ candidates determined by the workflow described in subsection 3.3.1 for both references were compared with each other and with Jagannathan and colleagues’ (Jagannathan et al., 2018) domestic horse variant list including 23.5 million SNVs. Candidates that were shared between lists and candidates unique to only one list were determined. At the same time, ‘de novo’ candidates produced by the PBT algorithm (see subsection 3.3.2) were compared to the finalized lists of both references and also to the Jagannathans and colleagues’ variant list. As coordinates of ‘de novo’ variants were different between reference versions, the positions needed to be converted to each other for the comparisons. This conversion was performed with the Remap (see Appendix Table A.A.1 for the version) algorithm provided by NCBI.

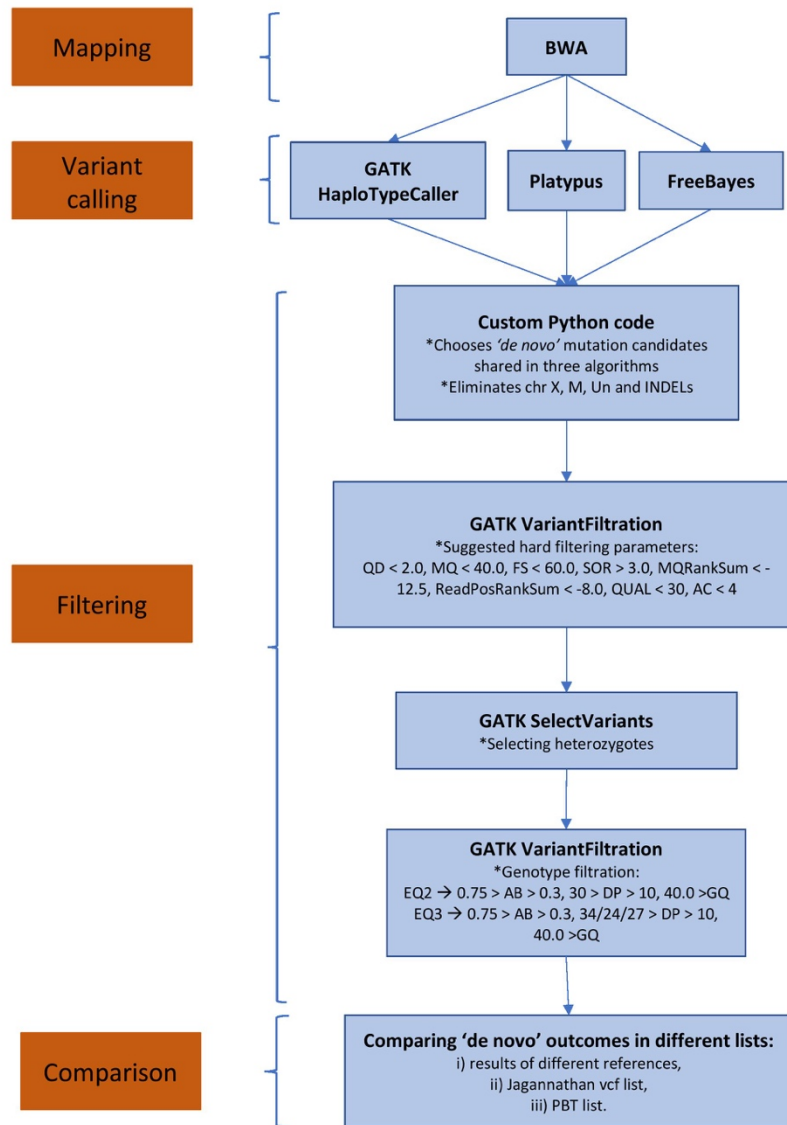


Figure 3.1: Workflow for detecting 'de novo' mutation candidates. There were four main parts which were mapping, variant calling, filtering and comparison with different variant lists. Main functions for filtering and their details are given in blue boxes.

3.4. Laboratory validation of EquCab2 candidates

All ‘de novo’ candidates predicted were checked in the IGV Genome Browser (see Table A.A.1). Based on this visual check, the most promising 50 ‘de novo’ candidates were chosen in total for laboratory validation by Sanger resequencing. The panel consisted of 20 ‘de novo’ variants in the Lipizzan trio (%39 of all Lipizzaner ‘de novo’ candidates), 14 in the Noriker trio (%24 of all Noriker ‘de novo’ candidates) and 16 in the Haflinger trio (%27 of all Haflinger candidates). Validation candidates are given in Table A.C.2 in Appendix C.

For each ‘de novo’ variant, flanking primers were designed for Sanger resequencing. Primers flanking the selected variants were designed using the web tool Primer3 (see Table A.A.1). Primer information is given in Appendix C, Table A.C.2.

Laboratory validation were made by Doris Rigler and Eva Michaelis at Institute of Animal Breeding and Genetics, University of Veterinary Medicine Vienna. Briefly, PCR amplification was performed using genomic DNA from each member of the respective trio (mother, father, son) as template. PCR reactions were carried out in a 20 µl volume containing 2µl genomic DNA (5 – 20 ng/µl), 0.5 µM of each primer, 1.5 mM MgCl and 1 x PCR buffer, 200 µM each dNTP and 0.1 U Taq DNA polymerase (AgrobioGen). The DNA was initially denatured at 95°C for 5 min, followed by 35 cycles of 30 s at 95°C, 30 s at annealing temperature (Supplementary Table S2) and 40 s at 72°C. After the 35 cycles, a final extension for 4 min at 72°C was performed. PCR products were visualized on a 2% agarose gel and purified using a QIAquick PCR purification kit. The concentration of the products was checked on a 2% agarose gel using the DNA ladder and then sent for Sanger sequencing to LGC genomics®. Results were visualized using the program Codon Code Aligner (see Table A.A.1). Laboratory validation were made by Doris Rigler and Eva Michaelis.

3.5. Mutation rate estimation

The number of truly validated ‘de novo’ mutations in EquCab2 mapped data were used to calculate an estimate of the mutation rate in one generation. To calculate the rate, the number of true ‘de novo’ mutations for each trio was divided by length of genome that was covered with the given thresholds in that trio (Data Analysis Part 3) as seen in the Equation 1. Overlapping genome sizes (bp) for each trio were calculated by multiplying overlapping genome percentages with two times of autosomal genome length (2,242,939,370 bp in EquCab2).

Mutation rate in one generation

$$(1) \quad = \frac{\text{\# of true de novo mutations}}{\% \text{ overlapping genome size} \times \text{genome length} \times 2}$$

3.6. Estimation of demographic history and runs of homozygosity

Pairwise Sequentially Markovian Coalescent (PSMC) analysis (Li & Durbin, 2011) was applied to estimate the demographic history of the breeds investigated. First, diploid consensus sequences were obtained from filtered (quality filtering and duplicate removal) versions of the BAM files (Data Analysis Part 2) mapped to the EquCab3 reference. SAMtools' 'mpileup' function with -C50 parameter and Bcftools (see Appendix A) were used to obtain these consensus sequences. While creating consensus sequences, a depth filter was applied to each trio again. The lower and upper thresholds were 10x to 2 times the mean trio coverage for each individual, respectively. In the next step, input files for the PSMC analysis were prepared with the 'q2psmcfa' function. The analysis was made with the suggested simulation parameters: "--N25 -t15 -r5 -p '4+25*2+4+6'", from the previous horse data analysis by Orlando and colleagues. Then, plots were drawn in R using the suggested generation time (8 years) and horse mutation rate (7.242e-09) from Orlando et al. (Orlando et al., 2013).

To estimate runs of homozygosity (ROH), variant files produced from EquCab3-mapped data by GATK HaplotypeCaller were used. Only the variants on the autosomal chromosomes were used in the analysis. ROHs were identified by PLINK (Purcell et al., 2007) (see Appendix A) with parameters: "--homozyg --homozyg-kb 500 --homozyg-snp 50 --homozyg-window-snp 50 --homozyg-window-het 3 --homozyg-window-missing 3 --homozyg-window-threshold 0.05 --homozyg-density 50".

CHAPTER 4

4.RESULTS

4.1.Primary data analysis and quality-based trimming

The total number of paired-end reads of each individual's raw data is given in Table 4.1. Whereas Haflinger BW-356 had the highest number of total reads in the data, Haflinger BW-357 had the lowest. The quality of raw reads was checked with FastQC and visualized with MultiQC. In Figure 4.1, the number of reads in different lanes are shown. Most of the Noriker and Haflinger lanes had a high amount of duplicates.

Table 4.1: The total number of reads in the raw fastq files for each individual. The same number of reads exist for both pairs.

Sample ID	Pedigree	Number of raw PE reads
111	Lip. Son	317,112,113
113	Lip. Dad	325,516,763
166	Lip. Mom	298,406,535
BW-352	Nor. Dad	365,143,936
BW-353	Nor. Mom	298,670,837
BW-354	Nor. Son	313,454,280
BW-355	Haf. Dad	313,514,763
BW-356	Haf. Mom	451,365,261
BW-357	Haf. Son	296,405,435

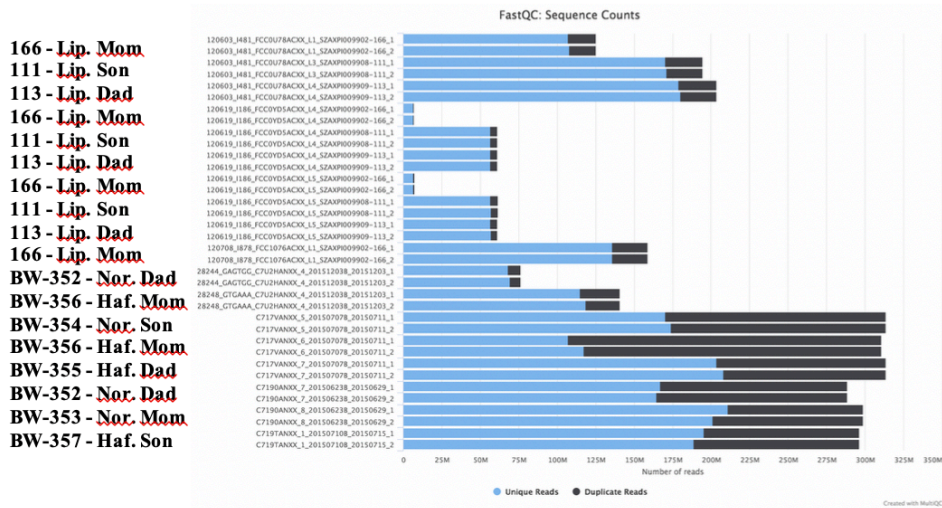


Figure 4.1: The number of paired-end reads in each lane. In the leftmost column, individual IDs and pedigree information for the lane are given. The proportion of duplicate (black) and unique (blue) reads for each lane are shown.

The number of reads and the mean Phred scores of different lanes are shown in Figure 4.2. While most of the reads increased for Noriker and Haflinger trios have very high mean Phred scores, in the Lipizzaner lanes the mean Phred score has a mode around 38 with few reads having qualities above 38.

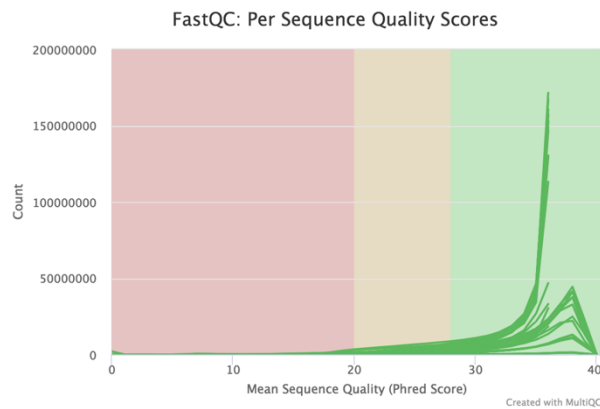


Figure 4.2: Mean Phred scores vs the number of reads of each lane. Most of the reads were above the mean Phred score of 30.

Figure 4.3 shows the distribution of each lane’s mean Phred score at each nucleotide position. In the untrimmed data, the Lipizzaner parents’ (113 and 166) reads had low Phred scores. Trimming parameters were thus chosen based on these low-quality lanes. After trimming, Phred scores were all over 30, throughout the read length. Length of the Lipizzaner reads were cut down to 81-86 base pairs (bp) from 90 bp, while Noriker and Haflinger reads were shortened to 99-113 bp from 120-125 bp.

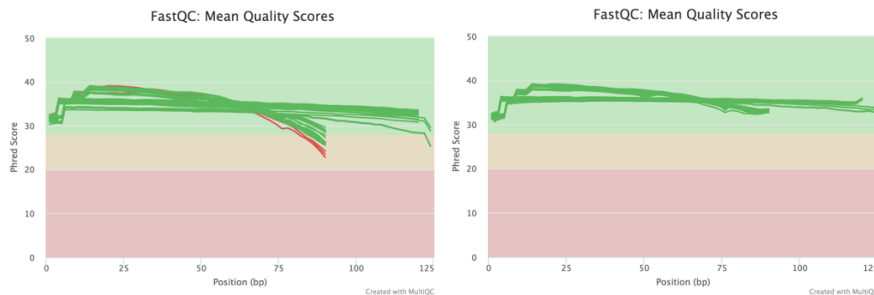


Figure 4.3: Mean Phred scores of each lane before (left) and after (right) trimming. After the trimming, reads with Phred score below 30 (red lines) for a position disappeared from data.

4.2. Mapping to the reference

The trimmed reads of each individual were mapped to the EquCab2 and EquCab3 reference genomes with BWA software. Different lanes that belonged to the same individual were merged into a single file. After that, the mapping files were filtered in order to remove unmapped reads and eliminate reads that were not properly paired and/or duplicated. In Table 4.2, the number of mapped reads in the raw data and in the filtered BAM files (i.e. properly paired and unique reads) are shown.

Table 4.2: The total number of reads for each individual, including both forward and reverse reads. BW-356 had the highest number of reads in the raw file, but after filtering this decreased dramatically, similar to the others. In the filtered data, the Lipizzan trio had the highest number of mapped reads.

Sample ID	Pedigree info	Total # of reads in raw files	Total # of mapped reads in filtered files (EQ2/EQ3)
111	Lip. Son	623366802	484898968/483215006
113	Lip. Dad	638708842	508612572/506977451
166	Lip. Mom	589312668	462960147/462505385
BW-352	Nor. Dad	699268926	303403168/303337894
BW-353	Nor. Mom	565506312	250130763/249834086
BW-354	Nor. Son	613144358	215904593/215968466
BW-355	Haf. Dad	613058170	291787760/291333599
BW-356	Haf. Mom	878454846	300332316/300064570
BW-357	Haf. Son	554469431	256835380/256756131

Mapping percentages of raw reads for different lanes are given in Table 4.3. Lipizzaners had the highest mapping rates (and properly paired read percentages) in both reference genomes while Norikers have the lowest.

Insert sizes of the final BAM files that were mapped to the EquCab3 reference were also calculated: Mean insert sizes were found as 475 bp in Lipizzaner, 308 bp in Noriker and 317 bp in Haflinger, and their medians were found as 476 bp, 306 bp and 316 bp respectively, in the finalized bam files.

4.3.Mapping coverage on autosomal chromosomes

Mean coverages on autosomal chromosomes of each individual are given in Table 4.4. All calculations were made for both reference genomes (EquCab 2 and 3). The Lipizzaner trio had the highest mean coverages, while Noriker individuals had the lowest.

Overlapping genomic regions which are in between threshold coverage values were calculated. The low and high thresholds were selected as 10 and 30, respectively, in EquCab2. In EquCab3, the low and high coverage thresholds were selected as 10 and twice the breed mean coverage, respectively. The lowest genomic size lying between these thresholds was calculated in the Noriker son (BW-354), whereas the highest was calculated in the Lipizzaner dad (113) for both references (see Table 4.4). Therefore, the Noriker trio had the lowest proportion of regions in the genome which were between the threshold values in all three trio-members and the Lipizzan trio had the highest proportion (Bold boxes in Table 4.4).

Table 4.3: Percentages of mapping and properly paired reads in each lane. Percentages were higher in the EquCab3 mapped data.

Sample ID	Pedigree info	Lane	% mapping (EQ2, EQ3)	% prop. paired (EQ2, EQ3)
111	Lip. Son	120619_I186_FCC0YD5ACXX_L4_SZA XPI009908	97.63, 98.04	94.53, 95.10
111	Lip. Son	120619_I186_FCC0YD5ACXX_L5_SZA XPI009908	97.72, 98.13	94.69, 95.27
111	Lip. Son	120603_I481_FCC0U78ACXX_L3_SZAX PI009908	98.04, 98.45	95.23, 95.82
113	Lip. Dad	120619_I186_FCC0YD5ACXX_L4_SZA XPI009909	97.68, 98.11	94.46, 95.07
113	Lip. Dad	120619_I186_FCC0YD5ACXX_L5_SZA XPI009909	97.76, 98.20	94.61, 95.23
113	Lip. Dad	120603_I481_FCC0U78ACXX_L4_SZAX PI009909	97.66, 98.09	94.42, 95.03
166	Lip. Mom	120619_I186_FCC0YD5ACXX_L4_SZA XPI009902	97.66, 98.10	93.92, 94.45
166	Lip. Mom	120708_I878_FCC1076ACXX_L1_SZAX PI009902	98.43, 98.89	95.30, 95.87
166	Lip. Mom	120603_I481_FCC0U78ACXX_L1_SZAX PI009902	98.12, 98.58	94.68, 95.25
166	Lip. Mom	120619_I186_FCC0YD5ACXX_L5_SZA XPI009902	97.76, 98.20	94.10, 94.62
BW-352	Nor. Dad	28244_GAGTGG_C7U2HANXX_4_2015 1203B_20151203	93.75, 94.15	87.51, 87.92
BW-352	Nor. Dad	C7190ANXX_7_20150623B_20150629	94.00, 94.57	85.66, 86.21
BW-353	Nor. Mom	C7190ANXX_8_20150623B_20150629	93.18, 93.70	85.57, 86.09
BW-354	Nor. Son	C717VANXX_5_20150707B_20150711	95.12, 95.67	88.17, 88.72
BW-355	Haf. Dad	C717VANXX_7_20150707B_20150711	94.96, 95.47	89.10, 89.62
BW-356	Haf. Mom	C717VANXX_6_20150707B_20150711	94.88, 95.44	89.24, 89.87
BW-356	Haf. Mom	28248_GTGAAA_C7U2HANXX_4_2015 1203B_20151203	94.20, 94.64	88.50, 88.99
BW-357	Haf. Son	C719TANXX_1_20150710B_20150715	95.06, 95.58	88.23, 88.76

Table 4.4: Mean coverages and the percentages among genome in between depth thresholds. While 10-30 depth thresholds were used in the EquCab2 data, 10 to 2 times breeds mean coverages of each trio were used in the EquCab3 mapped data.

Sample ID	Pedigree info	Mean Genome Coverage (EquCab2)	% of genome between 10-30 DP (EquCab2)	Overlapping genome size in 10-30 DP (EquCab2)	Mean Genome Coverage (EquCab3)	% of genome between 10-34/24/27 DP (EquCab3)	Overlapping genome size in 10-34/24/27 DP (EquCab3)
111	Lip. Son	17.32	88.17		17.16	88.11	
113	Lip. Dad	18.05	88.25	71.6	17.89	88.86	73.96
166	Lip. Mom	15.96	81.68		15.87	81.86	
BW-352	Nor. Dad	14.23	83.62		14.16	80.60	
BW-353	Nor. Mom	11.36	66.01	37.43	11.29	64.89	35.53
BW-354	Nor. Son	10.21	56.02		10.17	55.30	
BW-355	Haf. Dad	13.73	81.34		13.65	80.02	
BW-356	Haf. Mom	13.92	81.99	56.46	13.83	80.54	54.96
BW-357	Haf. Son	12.16	71.949		12.11	71.02	

4.4. ‘de novo’ mutation candidates

For detecting ‘de novo’ mutation candidates, the three main steps are mapping, variant calling, and filtering. These are followed by a comparison of the resulting variation lists (‘de novo’ candidate lists based on EquCab2 and EquCab3, Jagannathan’s variation panel, PBT ‘de novo’ candidates). Figure 4.4 shows the whole ‘de novo’ candidate detection workflow and the number of variants after each step. The same workflow was applied to both EquCab2 and EquCab3 mapped data, and both results were given in the figure respectively in each box.

4.4.1. Variant calls on EquCab2 and EquCab3

Three different algorithms were used to call variants in trios as shown in the variant calling part of Figure 4.4. GATK found the highest number of variants in all trios for both reference genomes, but the number of variants found in the updated horse genome release

EquCab3 was lower. The initial numbers given at the output of the variant calling step in Figure 4.4 include both SNVs and indels.

4.4.2. *Filtering variants to detect 'de novo' mutation candidates*

The filtering of the variants was started by finding 'de novo' candidates with a Python script (see Materials and Methods part 3.3.1) and eliminating variant calls on chromosomes X, M and Un, and also insertions and deletions (INDELs). At this point, the number of 'de novo' mutation candidates were highest in the results of the FreeBayes algorithm, among the three algorithms, for each trio and for both reference genomes (see Figure A.B.1 and Figure A.B.2 in Appendix B). In the next step, shared candidates were extracted between three algorithms and the highest number of common candidates were found between the GATK HaploTypeCaller and FreeBayes as seen in Appendix B, again for both reference genomes. On the other hand, the intersection of candidates between the three algorithms was highest in the Noriker trio and lowest in the Lipizzan trio.

Subsequently, variants were filtered based on different properties (heterozygosity, quality, etc.) as can be seen in the filtering part of Figure 4.4. At the end of the filtering, 51, 58 and 59 'de novo' candidates in EquCab2 mapped data, and 69, 45 and 55 candidates in EquCab3 mapped data were predicted in the Lippizaner, Noriker and Haflinger trios, respectively. Because different depth thresholds were applied to the variants at the last step of filtering, the minimum and the maximum number of resulting 'de novo' candidates were different in over trios and reference genomes. Detailed information of all 'de novo' candidates from the two reference genomes for the three trios is given in Table A.C.1 in Appendix C.

4.4.3. *Comparing finalized candidates from different lists*

In the last step of the workflow (Figure 4.4), finalized candidate lists from EquCab2 and EquCab3 and the common variant list from Jagannathan and colleagues (Jagannathan et al., 2018) which was created by 88 horses from EquCab3 reference genome were compared. Figure 4.5 shows the results of the comparison. Detailed information on 'de novo' SNPs is provided in the Table A.C.1. Candidates were also compared with the results from 'de novo' mutation detection algorithm PhaseByTransmission in GATK. Finalized 'de novo' candidates were examined in the IGV Genome Browser and chosen ones validated in the laboratory (see the subsection 4.5 titled *Laboratory Validation of 'de novo' Candidates* below).

a) Comparing to Jagannathan's common variant panel

38, 27, and 30 shared 'de novo' mutations were found between EquCab2 and EquCab3, respectively in Lipizzan, Noriker and Haflinger trios. Most of these 'de novo' mutation candidates were also found in the Jagannathan variation panel as seen in Figure 4.5. In particular, only 14 Lippizaner, 5 Noriker, and 12 Haflinger 'de novo' variants did not appear in Jagannathan's horse population variation list. Positions and detailed comparison results of all these mutations were given in Table A.C.1.

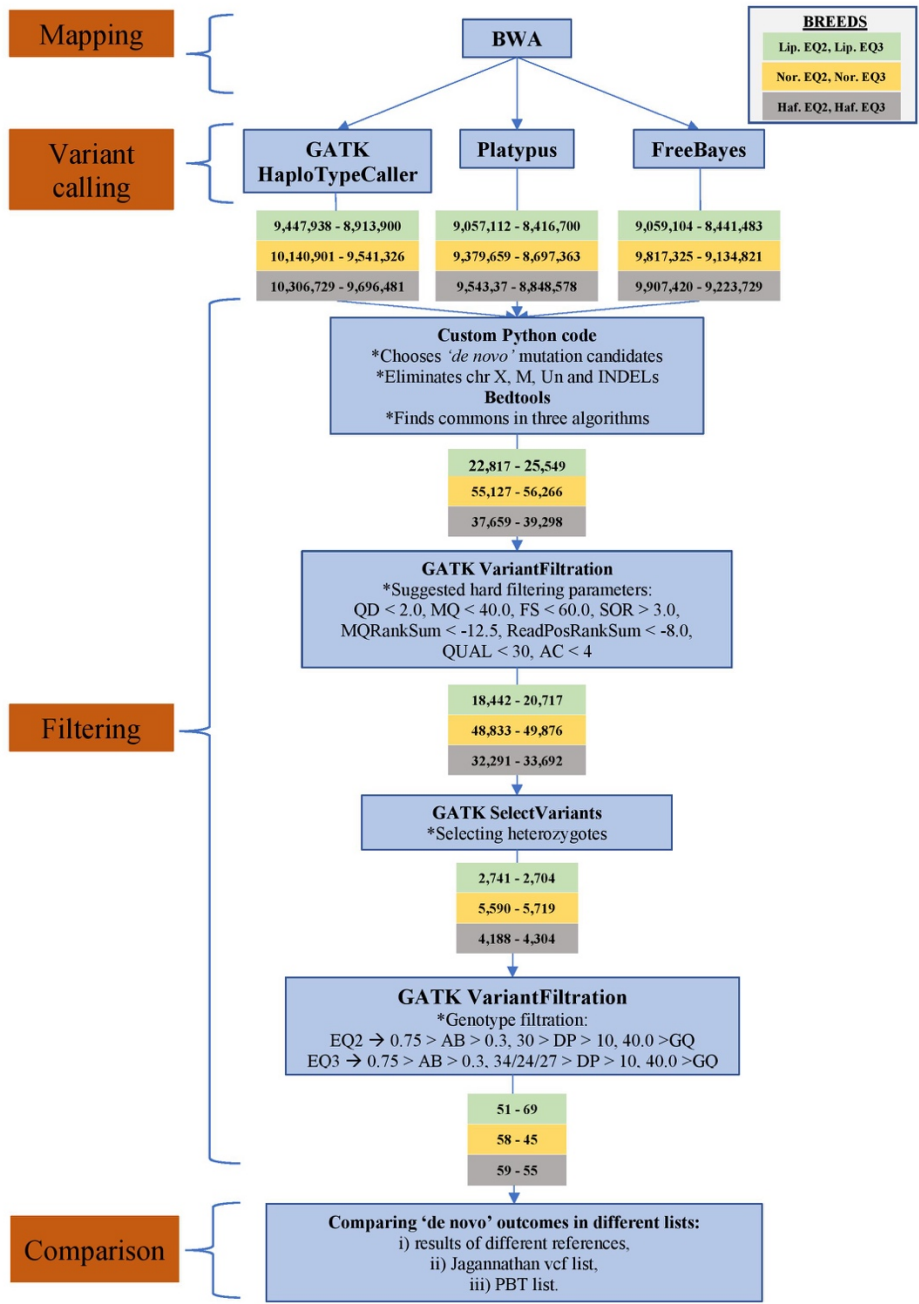


Figure 4.4: Shows analysis steps and the number of remaining mutations after each step. Blue boxes show the steps of the analysis, whereas green, yellow and grey boxes show the number of mutations in different breeds and different references after each step.

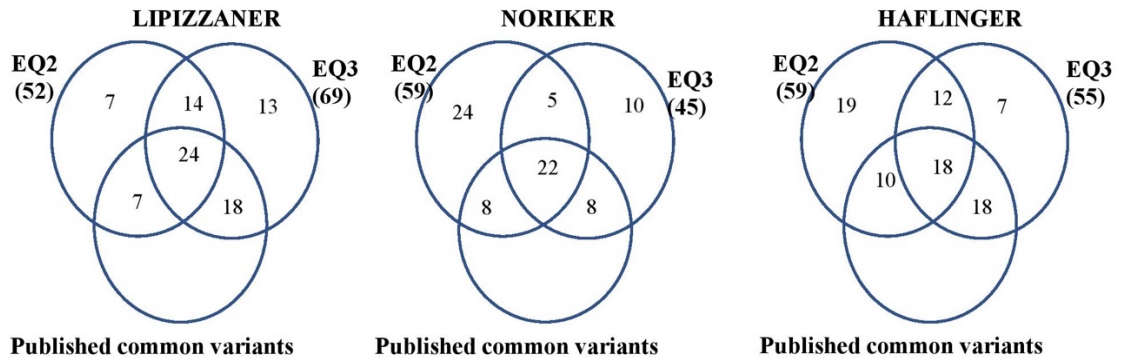


Figure 4.5: Comparison of finalized lists from the two reference genomes and published common variants. The highest number of shared mutations between the EquCab2 and EquCab3 genomes were found in the Lipizzaner trio. Total number of mutations in a set are given below the name of the set.

b) Comparing to the PhaseByTransmission result

‘De novo’ candidates in the Lippizaner trio were also predicted using the GATK PhaseByTransmission algorithm. Only 5 different candidates were detected at the intersection of the three different variant calling algorithms (GATK HaplotypeCaller, Freebayes, Platypus). These five variants were heterozygous in the offspring and homozygous for the identical allele in both parents. Positions and coverages of these five ‘de novo’ candidates are shown in Appendix C, Table A.C.2. Neither of these candidates were found in the EquCab2 or EquCab3 ‘de novo’ candidates generated in our custom workflow above, but three of them were found among Jagannathan's common variants.

4.5. Laboratory validation of ‘de novo’ candidates

Candidate ‘de novo’ mutations revealed after the filtering steps, as explained above, from EquCab2 mapped data (51 Lipizzaner-, 58 Noriker- and 59 Haflinger-variants) were visually checked in the IGV Genome Browser. Candidates that were located near other variants were eliminated and 50 candidate variants (20 in Lipizzaner, 14 in Noriker, 16 in Haflinger) were selected for independent lab validation. Detailed information of the validated variants is given in Table A.C.3, in Appendix C. For the chosen variants, locus-specific flanking primers were designed and a PCR product was amplified in mother (M), father (F) and son (S) and these were sequenced with Sanger technology.

The results of validation are shown in Table 4.5. 13 out of the 20 the Lipizzaner (around %65 of all tested candidates), 3 out of 14 the Noriker (around %21 of all tested candidates) and 5 out of 16 the Haflinger (around %31 of all tested candidates) candidates were successfully validated. In 5 Lipizzaner, 4 Noriker and 4 Haflinger candidates the putative ‘de novo’ allele was also detected in one of the parents. Some of the candidates could not be validated at all because Sanger sequencing did not produce good results in those positions (given in the *Missing* row in Table 4.5). Finally, some of the positions did not show the ‘de novo’ allele as observed in the NGS data. These are called ‘No SNV’ in

Table 4.5. IGV screenshots and Sanger validation results of a true ‘de novo’ mutation, a position that was not found as a variation in Sanger sequencing (i.e. ‘no SNV’) and a ‘de novo’ candidate that was also detected in the parents are, both shown in Appendix D.

Table 4.5: Results of laboratory validation. While some of the SNPs could not be detected with validation, some of them were also found in the parents. The highest proportion of true ‘de novo’ mutations were detected in the Lipizzaner trio.

	Lipizzaner	Noriker	Haflinger
Total number of validated SNVs	20	14	16
Missing (bad Sanger sequence)	1	1	2
SNV found also in parents	5	4	4
No SNV	1	6	5
Validated ‘<i>de novo</i>’ variant	13	3	5
% of truly validated candidates	~%65	~%21	~%31

Figure 4.6 shows the distribution of validation results and the comparison of three variant lists in addition to Figure 4.5. The 5 Lipizzaner, 2 Noriker and 3 Haflinger ‘de novo’ SNPs that were found also in the parents in the validation result were also found in the Jagannathan's variants. Besides, except one in Lipizzaner, all of the true positive ‘de novo’ mutations were detected in both EquCab2 and EquCab3 mapped data (see also Figure 4.6).

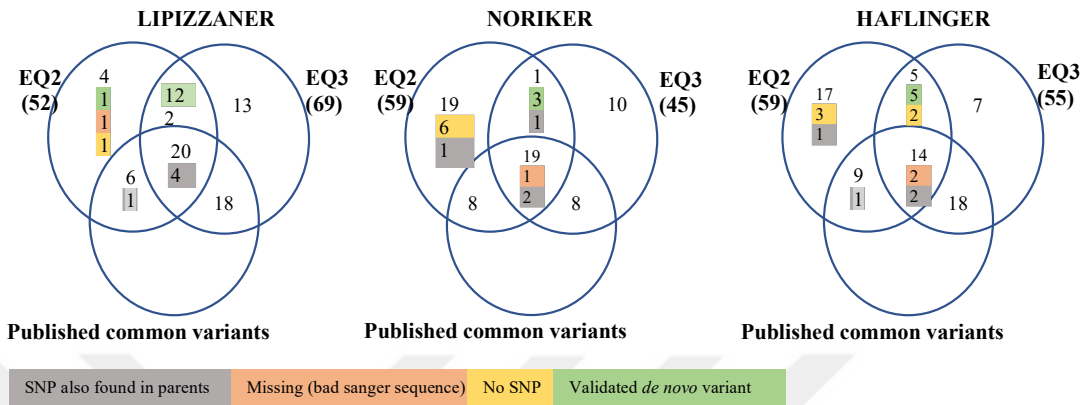


Figure 4.6: Comparison of three variant lists including validation results of EquCab2 mapped data. Grey label refers to SNPs which also determined in one of the parents in validation, orange label refers to bad Sanger sequenced positions, yellow label refers to variants not validated as SNP in Sanger results and green labels refers to validated ‘de novo’ variants. Except one, all true ‘de novo’ mutations are in the intersection of EquCab2 and EquCab3 mapped data and they are not seen in the Jagannathan’s variants.

4.6. ‘de novo’ mutation rate

The number of true ‘de novo’ mutations detected on the EquCab2 mapped data was used to calculate mutation rate in one generation (see Materials and Methods, part 6). To calculate mutation rates overlapping genome percentages (71.6 in Lipizzaner, 37.43 in Noriker and 56.46 in Haflinger) in the 10 to 30 depth threshold values were used as genome sizes and these revealed rates of $\sim 4e-09$ bp/gen, $\sim 1.7e-09$ bp/gen and $\sim 1.9e-09$ bp/gen in Lippizaner, Noriker and Haflinger trios, respectively. Detailed calculation steps are given in the Materials and Methods.

4.7. Estimation of demographic history and runs of homozygosity

To estimate the demographic history of the three populations, a Pairwise Sequentially Markovian Coalescent (PSMC) analysis was performed. The resulting PSMC graph is shown in Figure 4.7. All nine horses from three different breeds showed a similar pattern. There was a dramatic decrease in the effective population size around 500,000 years before present and the effective population size reached its maximum level around 120,000 years ago. The logarithmic version of the PSMC graph is given in Appendix E, Figure A.E.1.

The histograms showing the distribution of homozygous runs over lengths of runs are given in Figure 4.8. As evident from the figure, and as is expected, the number of runs decreased when the length of the run increased. The three Lippizaner horses had the highest mean number of homozygous runs for each histogram bin, whereas the Haflingers had the

lowest. In general, the distribution of the Haflinger and Noriker trios' runs of homozygosity were closer to each other, when compared with the Lippizaner trio.

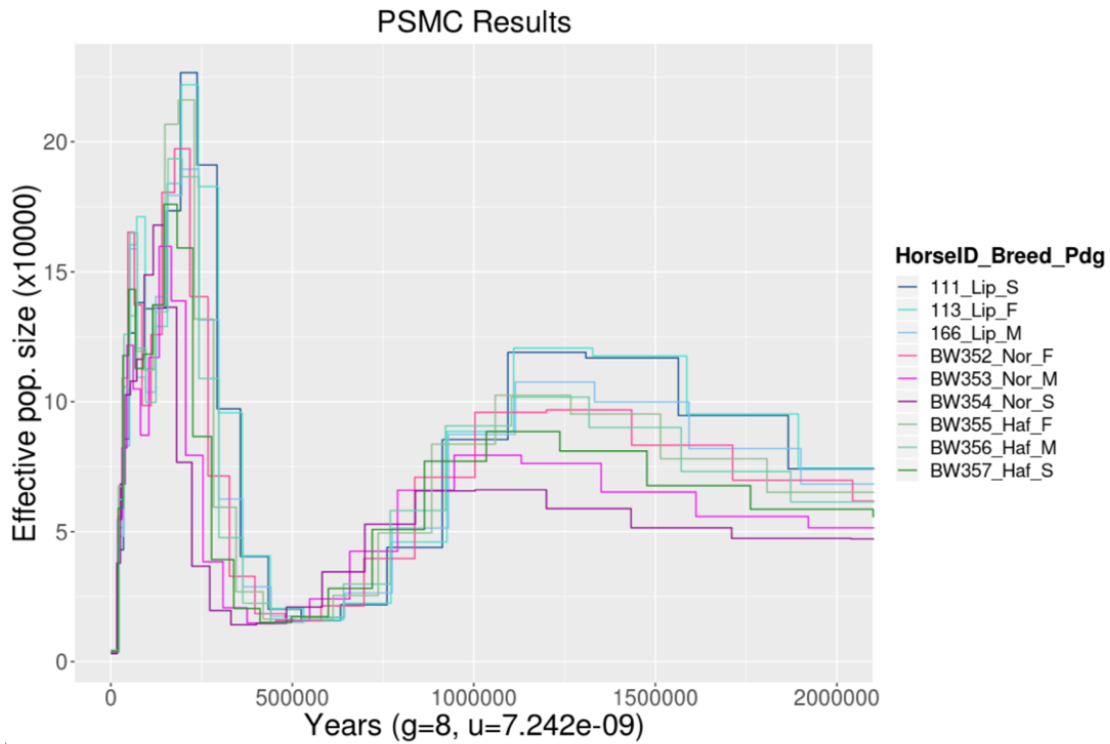


Figure 4.7: PSMC results for nine horses from three breeds as a function of time before present and effective population size. E.g., $2e+06$ in the x-axis is equal to 2 million years before present.

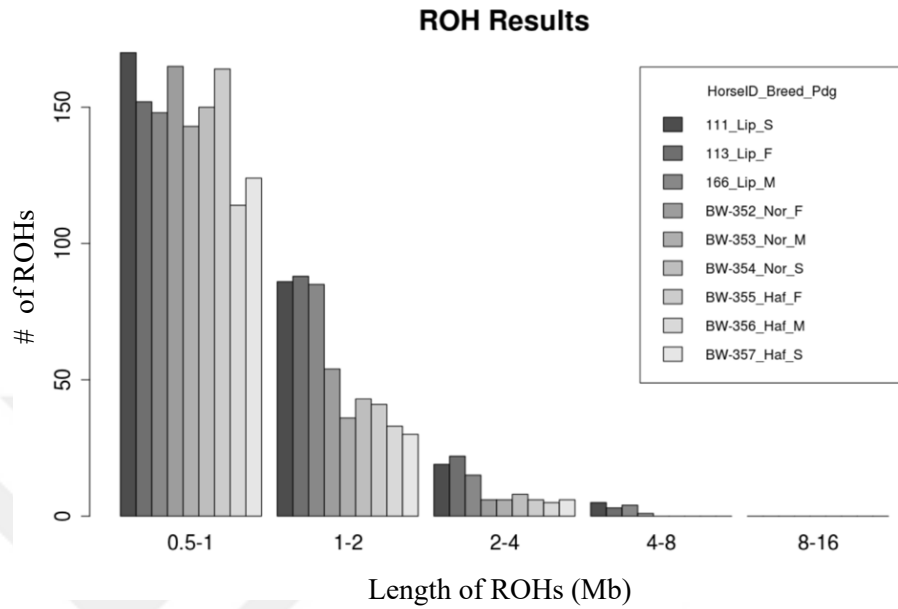


Figure 4.8: Distribution of homozygous runs for different length intervals. The x-axis refers to length of homozygous runs in megabases, the y-axis shows number of homozygous runs.



CHAPTER 5

5. DISCUSSION

In this thesis, we analyzed whole genome sequencing data of three horse trios. We designed a workflow to determine ‘de novo’ mutations accurately. We then calculated the mutation rate per generation with these ‘de novo’ mutations and compared this to the mutation rates from previous studies. Additionally, we made estimations regarding the demographic history of the three horse breeds by using PSMC and ROH analysis and compared results with previous studies.

5.1. Data, mapping, coverages and variant calling in different reference genomes

Some of the NGS sequencing lanes in the Noriker and Haflinger samples, produced by Vienna BioCenter (CSF) in 2015, had high numbers of duplicate reads. There are four main reasons for a duplicated read i) biological duplication (repeat regions), ii) PCR duplications, iii) optical duplicates which are the duplicated reads occur by accepting large clusters as two different clusters by mistake or by a local re-clustering of the original library polymer during bridge amplification, iv) ExAmp duplicates (Hadfield, 2016). Any of these can cause duplicate reads in Illumina sequencing. In our case, the main reason for having a high number of duplicates in some lanes in the Noriker and Haflinger data is assumed to be optical duplication, due to the sequencer used; HiSeq 2500 run mode HiSeq SBS V4. It has been suggested that it is more likely to have optical duplicates with this particular device and run mode (Hadfield, 2016).

When we compared mapping results between the EquCab2 and EquCab3 references, while the number of mapped reads after filtering were lower in EquCab3 mappings (Table 3-2), mapping percentages were increased in EquCab3 compared to EquCab2 mappings (Table 3-3). The increase in mapping ratio for the updated reference genome was also shown by Kalbfleisch and colleagues (Kalbfleisch et al., 2018). Besides mapping percentages, the number of properly paired reads also increased in the EquCab3 mappings. When we called variants with three different algorithms, we observed a decrease in the number of variant calls in the EquCab3 mapped data. This decrease in the number of

variants implies that false positive variants called in the earlier genome version (EquCab2) are now eliminated, as suggested in Guo et al., 2017.

The mean mapping coverage of each individual and overlapping genomic regions (i.e. regions where all members of a trio have coverage in between given thresholds) in each trio also differed between reference genomes (Table 4.4). The difference in the percentage of the overlapping genomic regions was mainly due to different covering thresholds being applied for the two different reference genomes (see 3.2.3). These changes in the threshold levels resulted in a wider depth range in the Lipizzaner trio in EquCab3 (threshold is 10 to 34 instead of 10 to 30 in EquCab2) and accordingly a larger percentage of the genome falling into this category in EquCab3. In contrast, a narrower range in EquCab3 in the Noriker (threshold is 10 to 24 instead of 10 to 30) and Haflinger (threshold is 10 to 27 instead of 10 to 30) lead to a decrease in percentages.

5.2. Variant calling with different algorithms and filtering

We performed variant calling with three different algorithms (GATK HC, Platypus, FreeBayes). GATK HC revealed the highest number of variants among them as seen in Figure 4.4. In the first step of filtering, we chose ‘de novo’ mutation candidates which show patterns as given in Table 2.2. Most variants were eliminated in this step as shown in Figure A.B.1 and A.B.2. This is expected because most variants are not expected to be ‘de novo’ candidates. In the next step, by eliminating the X, and Un chromosomes and the indels we lost almost half of the ‘de novo’ candidates. After this elimination, remaining candidates from three different algorithms were compared with each other to find shared ones. We observed that FreeBayes found the highest number of unique candidates which were not detected by any other algorithms, in the Noriker (83,593 in EQ2, 83,857 in EQ3) and Haflinger (56,279 in EQ2, 57,822 in EQ3) breeds (see Figures A.B.1 and A.B.2). GATK HC found the second-highest number of unique candidates in these trios. One reason for having the higher number of unique mutations called with different algorithms in Noriker and Haflinger could be the low sequencing coverage of some individuals in these trios. Tatsumoto et al., 2017 also suggests that false-positive variant prediction is more likely to occur in low coverage data. We also compared the intersection of the number of candidates detected by different algorithms pairwise and found the highest number of intersecting ‘de novo’ candidates among Freebayes and GATK HC, for both reference genomes and for each trio. Because this intersection did not include the candidates shared by all three of the algorithms, we speculate that most of these were false-positives, given that Platypus has a lower false-positive rate. We also found that the Lipizzan trio had the lowest number of mutations in the pairwise intersections of different algorithms as well as at the intersection of all three algorithms. This is consistent with the expectation of fewer false positives with the higher coverage of the Lipizzan data; fewer candidates that are more accurate are observed.

GATK Hard Filtering parameters were suggested by Summa et al. (De Summa et al., 2017) to detect the qualified variations among all variations. Around 15 % of the

candidates were filtered out in each trio and each reference with hard filtering. Selecting heterozygote ‘de novo’ candidates among all patterns (Table 2.2) also caused a dramatic decrease in the number of candidate ‘de novo’ mutations. To see a ‘de novo’ mutation having a homozygous allele pair in the offspring (see first four rows in Table 2.2), one of the parents should carry the same homozygous allele with the offspring and the other parent should be heterozygous at that position. This is a vanishingly low probability because it requires independent mutations in the offspring and one of the parents, or a back mutation (a mutation at the same position as a previous mutation that reverts the change of the former back to the reference allele), at that same position. Likewise, when the offspring is heterozygous (0/1) at a position, having homozygous alternative alleles (1/1) in the parents also means that a back mutation occurred in the position (see the last row of Table 2-2). Due to the extremely low probabilities of the aforementioned events, we speculate that only candidate ‘de novo’ patterns where the offspring is heterozygous (0/1), while the parents carry the homozygous reference allele (0/0) (see the fifth row of Table 2-2), have a chance to be a real ‘de novo’ mutation. We therefore focused on this pattern, applied filtering to choose this pattern among all ‘de novo’ patterns, and saw a dramatic decrease in the number of candidates. By eliminating the other patterns we speculate that we must have eliminated most of the false positive candidates (e.g. due to sequencing errors) in our data.

We finally filtered candidates for allele balance and depth, as suggested in several studies (Jónsson et al., 2017; Tatsumoto et al., 2017). In EquCab2 data, we filtered for ‘de novo’ candidates with 10 to 30 read depth values and we got the lowest number of candidates in Lipizzaner trio (51), whereas higher numbers in Noriker (58) and Haflinger (59) trios. Because the lowest overlapping regions between 10 to 30 depth values were found in the Noriker, followed by the Haflinger trios, we speculate that a higher number of false positives in the ‘de novo’ candidate list of Noriker and Haflinger trios caused this observation. We therefore changed the depth thresholds from 10 to two times mean coverages of the respective trios when analyzing the EquCab3 mapped data. Because mean coverage is lower in Noriker and Haflinger trios, their upper thresholds were thus also lower (24 for Noriker, 27 for Haflinger), while Lipizzaner had a higher upper threshold (34). These changes in the threshold caused an increase in the number of ‘de novo’ candidates in the Lipizzaner (69) list and decrease in Noriker (45) and Haflinger (55) trios. This shows that setting the upper limit as a function of the mean lowers the probability of false positives (see Figure 4.5) especially in low covered trios (Noriker and Haflinger). Keeping depth filtering as a function of mean was also suggested by Li and Wren (Li & Wren, 2014) When we compared the number of ‘de novo’ candidates with the numbers in other mammalian studies (Conrad et al., 2011; Jónsson et al., 2017; Tatsumoto et al., 2017), we saw that they were comparable.

5.3. Interpretation of ‘de novo’ candidates and Sanger validation results

We compared the candidate lists from EquCab2 and EquCab3 mapped data and found the highest number of shared mutations in Lipizzaner (38) and the lowest in Noriker (27)

(Figure 4.5). We expected that intersecting candidates on two independent callings using different reference genomes were the best candidates for true ‘de novo’ mutations. Besides, to get a feeling how many of false positive candidates are still in our list, we compared the candidates to a recently published horse variation dataset given in Jagannathan et al. (Figure 4.5) (Jagannathan et al., 2018). For our purposes, the Jagannathan panel serves as a database for standing variation in the Central European horse population. We found a remarkable overlap especially in the Noriker and Haflinger ‘de novos’ as shown in the Figure 4.5. Possible explanations for this observation are sequencing errors or low coverage sequencing of parents’ data. Because heterozygosity in one parent was not detected with Illumina sequencing, some variants falsely give a ‘de novo’ mutation pattern after filtering. We saw this ratio was higher in the Noriker (22 out of the 27 ‘denovo’ variants also in Jagannathan dataset) data than in the Lipizzaner (24 out of 38) and Haflinger (18 out of 30) data. These results support our hypothesis of a higher amount of false positives in the lower coverage Noriker trio.

We performed Sanger resequencing for a selected set of ‘de novo’ mutations after checking all candidates in the IGV Genome Browser for both parents and offspring. Results of laboratory validation allowed us, on the one hand, to infer the validity and true number of our ‘de novo’ predictions, and on the other hand, they illuminate possible scenarios leading to false positive candidates. For example, i) Some could be sequencing errors in the offspring and we called them ‘no SNP’ in the validation results. ii) The putative ‘de novo’ allele was also detected in one of the parents with Sanger sequencing. These positions were most probably not called with Illumina sequencing because of the low coverage. We called these ‘SNP also in parent’. iii) ‘de novo’ candidates that could not be confirmed with Sanger sequences, due to inconclusive Sanger results, were classified as ‘bad Sanger sequence’. Such errors occur when the position is in for example in a duplicated regions in the genome, as suggested in the Tatsumoto et al., or the PCR amplicon for Sanger sequencing was not locus specific; iv) Finally, true ‘de novo’ mutations that were validated successfully and the ‘de novo’ allele was detected only in the offspring by both NGS and Sanger sequencing.

Validation results (Table 4.5) were consistent with the data quality. Ratio of truly validated mutations was 13/20, 3/14 and 5/16 in Lipizzaner, Noriker and Haflinger trios respectively. The results clearly showed that, sufficient read coverage is essential to detect true positive mutations. We expected this result because we had the of the highest mean coverage in the Lipizzaner (~17x) and the lowest in the Noriker (~12x) data. Tatsumoto et al. suggested that even 30x and 60x coverages are not enough to detect all ‘de novo’ mutations in a trio data (Tatsumoto et al., 2017). Although our data was not nearly that deeply covered, with our custom filters we have reduced the number of false positives and detected a healthy number of ‘de novo’ mutations which are true positive.

We make two inferences from our results at this point: First, Lipizzaner data coverage is at the edge of the coverage necessary to reliably detect a sufficient number of true ‘de novo’ mutations. Second, our workflow still gives partially accurate results, in spite of the

low coverage. To sum up, having genomic data with at least 17x mean coverage and applying well-adjusted depth thresholds could be the most important steps in finding true ‘de novo’ mutations.

Number of candidates and true positives also correlate with the size of the genomic regions that were considered for analysis. The Lipizzaner trio had the largest genomic area (71,60% in EquCab2) within the thresholds, whereas Noriker had the lowest (37,43% in EquCab2). Based on this, we expect to find more candidates and true ‘de novos’ in Lipizzaner, and less in Noriker. Figure 5.1 shows the relation between scanned regions (1.605 x 10⁹ Mb in Lipizzaner, 0.839 x 10⁹Mb in Noriker and 1.266 x 10⁹ Mb in Haflinger) and true positive mutations (13 in Lipizzaner, 3 in Noriker and 5 in Haflinger).

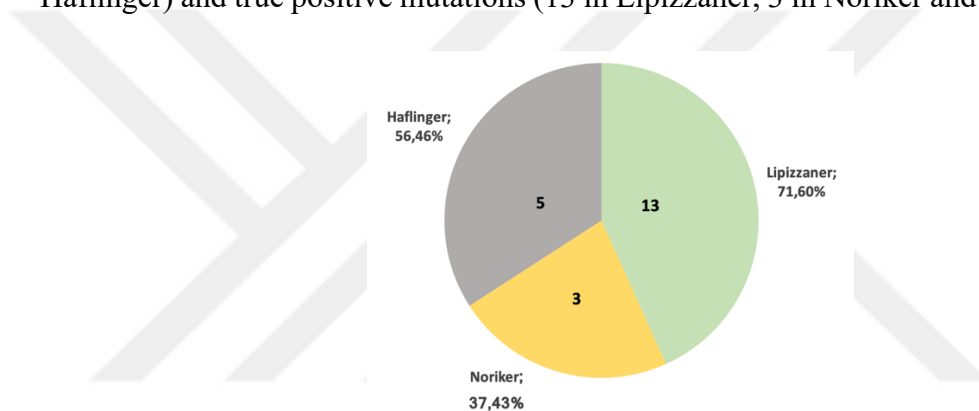


Figure 5.1: Percentages of overlapping genomic regions that were covered between 10-30 in EquCab2 in the respective trio and number of true ‘de novo’ mutations.

We also checked the distribution of the validation results among EquCab2, EquCab3 and the variant dataset from Jagannathan et. al. We found that all of true ‘de novo’ mutations, except one, in Lipizzaner were detected in both EquCab2 and EquCab3 mapped data but not in the Jagannathan variation panel, as expected (Figure 4.6). We found that many of the candidates which were classified as ‘SNP also found in parents’ were also in the Jagannathan data. Seeing these variants already in the Jagannathan data is further proof that Illumina sequencing missed heterozygous alleles in the parents due to very low sequencing coverage. None of the ‘no SNP’ positions were found in the Jagannathan’s variation list. This was expected and is another confirmation that these positions are sequencing errors in the offspring.

5.4.Mutation rate differences

We calculated a ‘de novo’ mutation rate to be 4.05 per billion sites per generation for the Lippizaner, 1.79 for the Noriker, and 1.97 for the Haflinger trio, by dividing number of true positive ‘de novo’ mutations detected in each trio with 2 times overlapping genomic area of the respective trio in 10-30 coverage thresholds. We compared these ratios with human, chimpanzee and mouse rates which were calculated with the same equations.

When we compare these rates with the germ line ‘de novo’ mutation rate of human which is $1.29e-08$ (Jónsson et al., 2017) our rates are almost 3 to 6 times smaller than the human rate. In the chimpanzee case the mutation rate was calculated as $1.48e-08$ (Tatsumoto et al., 2017) and our Lippizzan rate was almost 4, Noriker rate was almost 9, and Haflinger rate was almost 8 times smaller than chimpanzee rate. When we compared the estimated horse rates with the mouse mutation rate estimates, ranging from $3.9e-09$ (Lindsay et al., 2016) to $5.4e-09$ bp/gen (Uchimura et al., 2015), our rates appeared comparable with them. Besides, when we compared the estimated rates with the mutation rate calculated for horses by phylogenetic distance method which is $7.2e-09$ bp/gen (Orlando et al., 2013), we could say that we estimate the rate at least in the same decimals. One possible reason of the difference between phylogenetic and ‘de novo’ rates could be the false negative ‘de novo’ mutations that we missed during the analysis because of our data quality. However, other explanations are also possible (see Introduction).

When we compared different mutation rates between our three horse trios, we found the highest rates in the Lipizzaner whereas Noriker and Haflinger rates were lower and closer to each other. We speculate that the main reason for this difference is again the quality of the sequencing data. We had the highest coverage in the Lipizzaner trio, and thus the most accurate ‘de novo’ mutation rate is the one calculated from Lipizzaner data. Other possibilities of getting different rates could be fathers’ age (Francioli et al., 2014; Kong et al., 2012) and background effects differing between families (Conrad et al., 2011).

5.5. Estimations on population history

5.5.1. PSMC

To estimate the demographic history of *Equus caballus*, we performed PSMC analysis. Results of the analysis revealed patterns similar to the ones presented by Orlando et al., 2013 and Schubert et al., 2014. The horse effective population size reached a minimum value between 100 to 130 thousand years (kyr) before present in our data. Orlando et al. found the same decrease 125 kyr before present, and they suggested that the Last Interglacial Period was the reason for this decrease. This decrease is followed by a peak in the effective population size in our findings, as well as in Orlando et al., 2013. This peak is thought to have developed due to the environmental changes after the Last Glacial Maximum (Lorenzen et al., 2011). However, although this timeline is consistent with our findings, there are still some differences in the timeline vs. effective population size patterns of our nine horses. One reason for this could be, again, the low mean coverage of our data. Nadachowska-Brzyska and colleagues have suggested that the PSMC method is not able to produce reliable models under 18x mean coverage, and our mean coverages in EquCab3 mapped data ranged between 10.17 and 17.89 (Nadachowska-Brzyska et al., 2016). The difference between mean coverages could also explain the differences in the models of different individuals. Despite these differences, we suggested that taking the regions in between 10 to 2 times mean coverage threshold (Li & Wren, 2014) provides

consistent pattern among individuals and with the other studies (Orlando et al., 2013; Schubert et al., 2014).

5.5.2. *Runs of homozygosity analysis (ROH)*

We also performed a ‘runs of homozygosity’ analysis on our data to estimate population histories of the three different Austrian breeds in our study. Ceballos and colleagues suggested that inbred populations have a higher number of longer ROHs than outbred populations. They also suggested that if the population has a high number of shorter runs and along with some longer ROHs, it is possible that the population is both inbred and bottlenecked (Ceballos et al., 2018). When we interpreted our results from this point of view, we saw that the Lipizzaner trio is more inbred than the Noriker and Haflinger trios. Previous studies have found higher inbreeding in Austrian Lipizzaners with respect to other Lipizzaners (Grilz-Seger et al., 2019), and more inbreeding in Lipizzaners as compared to Norikers (Druml et al., 2009). Additionally, no appreciable difference between Lipizzaner and Haflinger populations in inbreeding was reported (Grilz-Seger et al., 2019).

Our ROH results which showed a low number of long ROHs (>4Mb) and high number of short ROHs (<2Mb) in Noriker and Haflinger trios, suggest less inbreeding in recent generations when compared with Lipizzaners (Ceballos et al., 2018). The conclusion of more inbreeding in Lipizzaner compared to Noriker is also supported by Druml et al., 2009. On the other hand, more inbreeding in Lipizzaner than Haflinger was not reported previously. This could be an effect of coverage differences in our data or might reflect variation within these breeds.

5.6. Future work and conclusion

We only validated candidate mutations from EquCab2 mapped data, but in addition we defined new possible candidate ‘de novo’ mutations called on EquCab3 only, which were not found in Jagannathan’s data set (green labeled candidates in Appendix Table A.C.1). These ‘de novo’ candidates should be validated as well. While validating them in the future, besides taking the blood sample used for Illumina sequencing as a source for Sanger validation, another somatic tissue also could be used to distinguish true germ line ‘de novo’ mutations from somatic ‘de novo’ mutations. Only if the same ‘de novo’ mutations are validated in the other somatic cells, we can be definitely sure that they are germ line and not somatic cell mutations (Tatsumoto et al., 2017).

The statistical power of ‘de novo’ mutation detection workflows should also be measured in terms of false negative rate. To calculate false negative rate, we would need to estimate ‘de novo’ mutation candidates with another data set which have also validated true ‘de novo’ mutations. By trying our workflow on such a more deeply sequenced data we could have an estimate on false negative rate, and thus could obtain a more accurate mutation rate per generation for horse. For this aim we can analyze publicly available genomic

datasets, which include truly validated ‘de novo’ mutations (Francioli et al., 2014), in order to test its reliability. Another option could be analyzing the 'Genome in a Bottle' data set which includes deep coverage (~300x) human trio data (Zook et al., 2016), and we could compare verified ‘de novo’ mutations from this data set with ‘de novo’ mutations estimated from our workflow (Zook et al., 2018) and using the same coverages as in the horse data.

The demographic analysis could also be enriched, e.g. by calculating the inbreeding coefficient (F_{ROH}) from ROH data, by dividing average length of ROHs to autosomal genome size (McQuillan et al., 2008), and comparing these estimates with other studies' outcomes (Druml et al., 2009; Grilz-Seger et al., 2019).

In conclusion, our data is at the very low-end coverage compared to many other studies for 'de novo' mutation estimation. Nevertheless, we were able to analyze this data to get as much information out of it as possible. We were able to detect a significant number of the true ‘de novo’ mutations from trio data with our workflow, and this was the achievement of our workflow for ‘de novo’ mutation detection. On the other hand, PSMC and ROH results were consistent with previous studies in most cases. Our study thus provided insight into the minimum data quality needed for such analyses.

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APPENDICES

APPENDIX A

USED TOOLS

Table A.A.1: Programs and their versions that used in the analysis.

Program	Version
SAMtools	1.4
Bedtools	2.25.0
FastQC	0.11.5
MultiQC	v1.7.dev0
Trimmomatic	0.36
Picard Tools	1.119
R	3.2
GATK	3.8-0
Freebayes	v0.9.10-3-g47a713e
Python	2.7, 3
Remap	2.1
IGV	2.4.10
Primer3	v0.4.0
Codon Code Aligner	3.0.1
Bcftools	1.9
Vcftools	0.1.13
Plink	1.07

Table A.A.2: Usage purposes and command line usages of each tool.

Tool/Algorithm	Process	Command line
SAMtools	Sorting	samtools sort @2 input.bam -o output.bam
BEDtools	Converting bam file to fastq file	bedtools bamtofastq -i input.bam -fq read1.fq -fq2 read.fq
FastQC	FatsQC analysis	fastqc -o . -f fastq input.fq.gz -t 5
Trimmomatic	Trimming data	trimmomatic-0.36.jar PE -threads 6 read1.fq.gz read2.fq.gz read1_paired.fq.gz read1_unpaired.fq.gz read2_paired.fq.gz read2_unpaired.fq.gz TRAILING:10 MINLEN:50 SLIDINGWINDOW:5:20
MultiQC	Reporting FastQC results	multiqc .
bwa	Indexing reference	bwa index -a bwtsv reference.fa
bwa	Mapping files to the reference	bwa mem -R '@RG\tID:laneID\tSM:horseID' -M -t 8 reference.fa read1.fq.gz read2.fq.gz > mappedFile.sam
SAMtools	Convert sam to bam	samtools view -b -S mappedFile.sam > mappedFile.bam
SAMtools	Merging different lanes	samtools merge merged.bam lane1.bam lane2.bam lane3.bam
SAMtools	Taking properly paired reads	samtools view -B -F 4 -f 2 -h input.bam > output.bam
Picard Tools	Removing duplicates	java -jar MarkDuplicates.jar I=input.bam O=output.bam METRICS_FILE=metrics.txt REMOVE_DUPLICATES=TRUE
SAMtools	Filtering reads for quality	samtools view -q 20 -b input.bam > output.bam
Picard Tools	Calculating insert sizes	java -jar CollectInsertSizeMetrics.jar I=input.bam O=insert_size_metrics.txt H=insert_size_histogram.pdf M=0.5
SAMtools	Indexing bam files	samtools index input.bam

Bedtools	Calculating genomic coverages	<code>bedtools genomecov -ibam input.bam -g reference.fa > output.txt</code>
Bedtools	Calculating positional coverages	<code>bedtools genomecov -ibam input.bam -g reference.fa -d > output.txt</code>
GATK HaploTypeCaller	Calling variants	<code>java -jar GenomeAnalysisTK.jar -T HaplotypeCaller --(fix_misencoded_quality_scores) -R reference.fa -I son.bam -I father.bam -I mother.bam -o variants.vcf</code>
Platypus	Calling variants	<code>python Platypus.py callVariants --bamFiles=son.bam,father.bam,mother.bam --refFile=reference.fa --output=variants.vcf</code>
Freebayes	Calling variants	<code>freebayes -f reference.fa -L bamList.txt > variants.vcf</code>
Bedtools	Intersecting bed files	<code>Bedtools intersect -a file1.bed -b file2.bed > file3.bed</code>
GATK VariantAnnotator	Annotating variants	<code>java -jar GenomeAnalysisTK.jar -T VariantAnnotator --(fix_misencoded_quality_scores) -R reference.fa -I trioBams.list -V variants.vcf -o annotatedvaariats.vcf -A AlleleBalanceBySample</code>
GATK VariantFiltration	Filtering variants in vcf	<code>java -jar GenomeAnalysisTK.jar -T VariantFiltration -R reference.fa -V variants.vcf --filterExpression "QD < 2.0 MQ < 40.0 FS > 60.0 SOR > 3.0 MQRankSum < -12.5 ReadPosRankSum < -8.0 QUAL < 30 AC > 4 " --filterName "my_snp_filter -o output.vcf</code>
GATK SelectVariants	Selecting heterozygous variants	<code>java -jar GenomeAnalysisTK.jar -T SelectVariants -R reference.fa -V variants.vcf -select 'vc.getGenotype("horseID").isHet()' -o output.vcf</code>

GATK VariantFiltration	Filtering genotype of different individuals in vcf	<pre> java -jar GenomeAnalysisTK.jar -T VariantFiltration -R reference.fa -V variants.vcf --genotypeFilterExpression "AB < 0.3 AB > 0.75" --genotypeFilterName "AB_filter" --genotypeFilterExpression "DP > 34/24/27 DP < 10" --genotypeFilterName "DP_filter" --genotypeFilterExpression "GQ < 40.0" --genotypeFilterName "GQ_filter" -o output.vcf </pre>
GATK PhaseByTransmission	Calling de novo candidates	<pre> java -jar GenomeAnalysisTK.jar -T PhaseByTransmission -R reference.fa -V variants.vcf --MendelianViolationsFile mendelianViolations - ped trio.ped -o output.vcf </pre>
SAMtools, Bcftools	Preparing consensus sequences for psmc	<pre> samtools mpileup -C50 -uf reference.fa input.bam bcftools call -c - vcftools.pl vcf2fq -d 10 -D 34/24/27 gzip > output.fq.gz </pre>
PSMC	Converting fastq to psmcfa	<pre> fq2psmcfa -q20 file.fq.gz > file.psmcfa </pre>
PSMC	Psmc analysis	<pre> psmc -N25 -t15 -r5 -p "4+25*2+4+6" -o output.psmc input.psmcfa </pre>
Vcftools	Converting vcf top link format	<pre> vcftools --vcf input.vcf --out output --plink </pre>
Plink	Converting file to Plink's bed format	<pre> plink --noweb --horse --file input --out output -- make-bed </pre>
Plink	Roh analysis	<pre> plink --noweb --horse --bfile inputFile --homozyg -- homozyg-kb 500 --homozyg-snp 50 --homozyg- window-snp 50 --homozyg-window-het 3 -- homozyg-window-missing 3 --homozyg-window- threshold 0.05 --homozyg-density 50 </pre>

APPENDIX B

FILTERING DETAILS

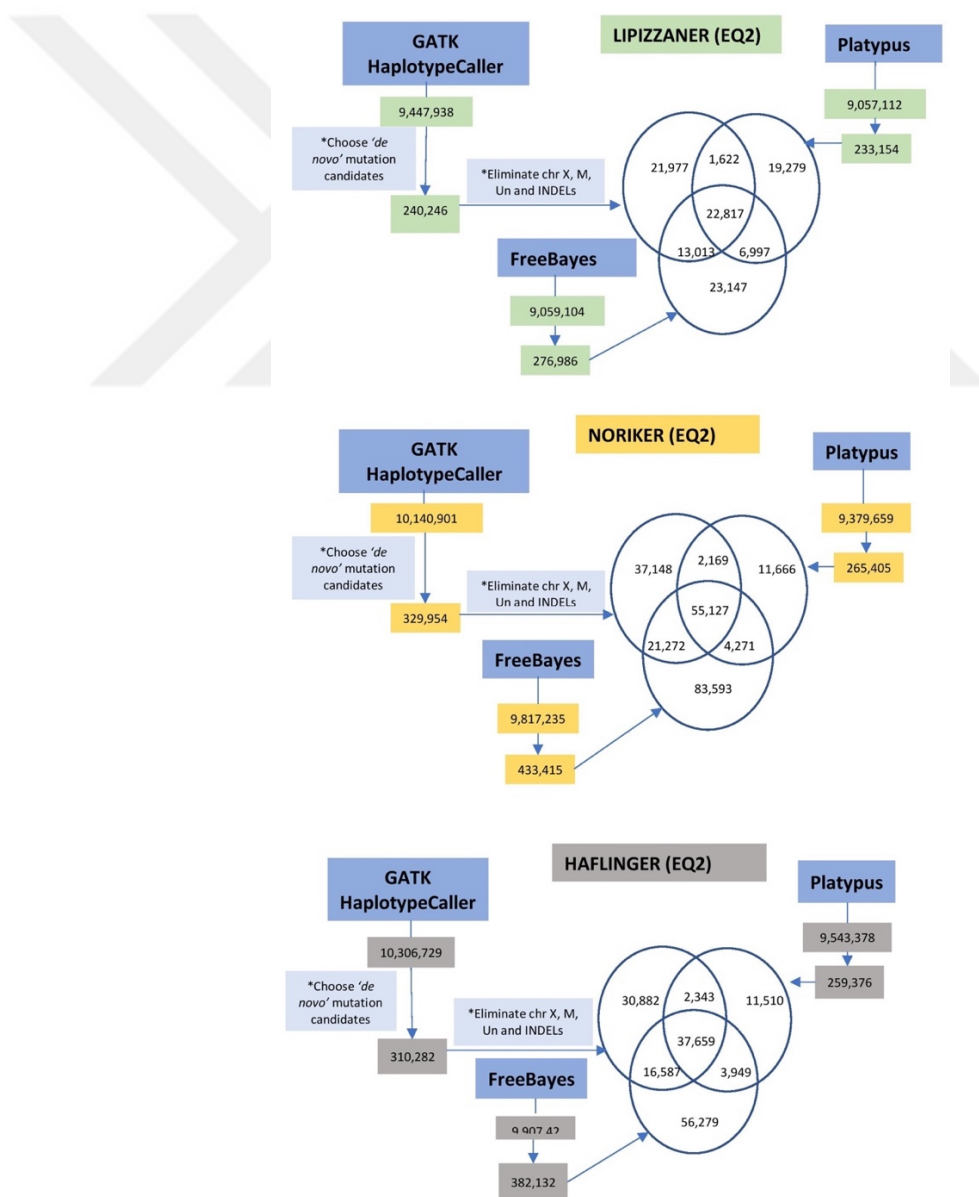


Figure A.B.1: Detailed results of first filtering step in Figure 4.4 for EQ2 mapped data.

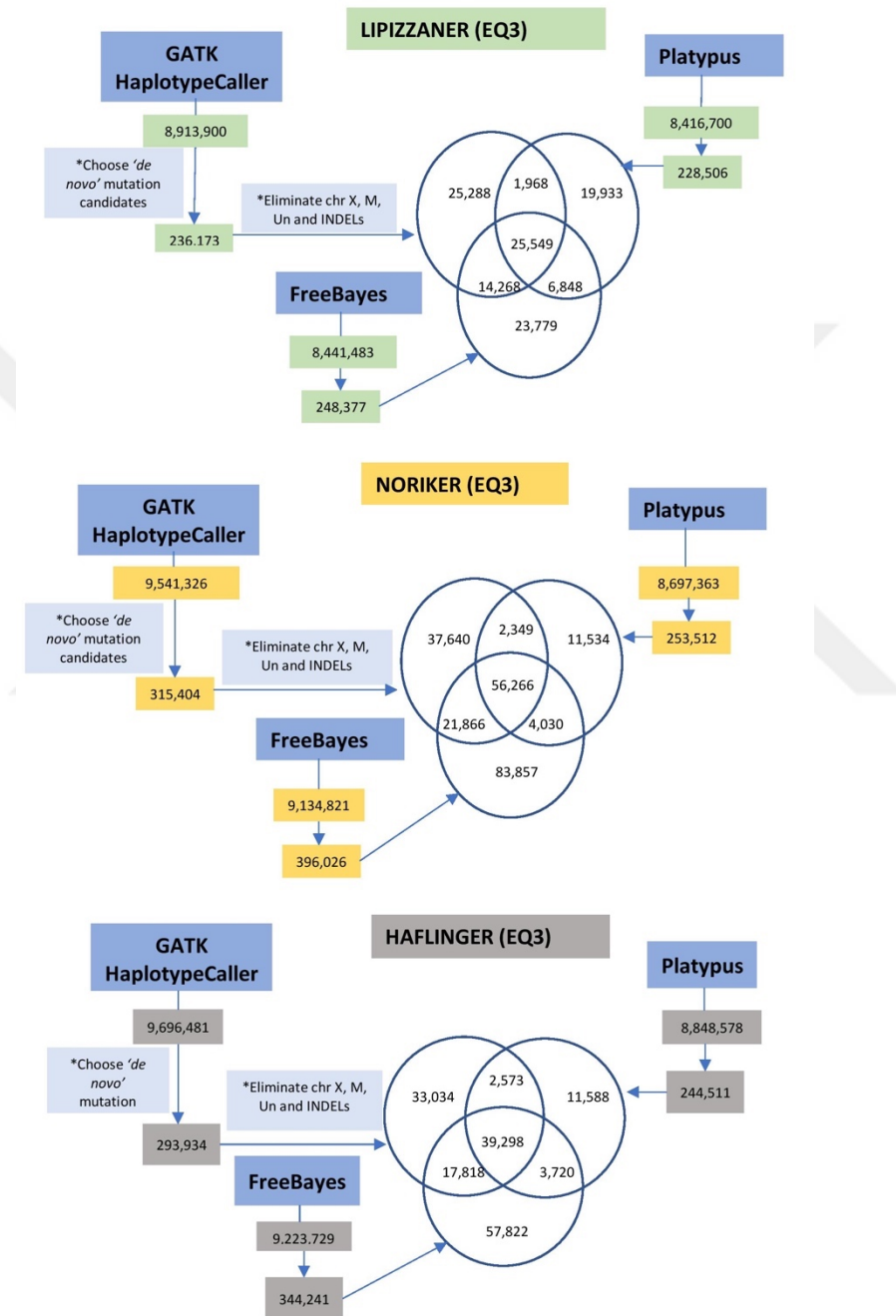


Figure A.B.2: Detailed results of first filtering step in Figure 4.4 for EQ3 mapped data.

APPENDIX C

DETAILED INFORMATION ABOUT DE NOVO CANDIDATES

Table A.C.1: Position and comparison information of all candidate ‘de novo’ mutations. Some of the reads mapped in one genome version (for example EquCab2) were mapped more than one position in the other genome version (EquCab3). Such mutations (red labeled) were detected in the multiple positions when the position converted to the other reference version. The mutations in the fully red written rows cause inconsistency between comparison (EQ2, EQ3 and Jagannathan’s panel) results. The chosen mutations for lab validation were indicated in the last column. Green labeled candidates were chosen for future validation depending on their coexistence in both reference mapped data and not existence in Jagannathan data.

TRIO	EQ2 chr	EQ2 pos	EQ3 chr	EQ3 pos	Ref	Alt	Found or not found in EQ3	In Jagannathan data	Sanger validation done
LIP	chr1	15859530	chr1	15978254	C	G	found	no	yes
LIP	chr1	15864504	chr1	15983228	G	T	found	no	yes
LIP	chr1	68664504	chr1	69214531	A	C	found	no	yes
LIP	chr10	82141638	chr10	83318763	G	A	found	no	yes
LIP	chr13	27768579	chr13	28928832	T	G	found	no	yes
LIP	chr19	43987271	chr19	46744475	C	T	found	no	yes
LIP	chr2	49697936	chr2	49938684	G	T	found	no	yes
LIP	chr2	69806759	chr2	70279783	A	C	found	no	yes
LIP	chr4	96806258	chr4	96948867	C	T	found	no	yes
LIP	chr5	95223175	chr5	92308816	G	A	found	no	yes
LIP	chr8	29308865	chr8	31918931	G	A	found	no	yes

LIP	chr8	83490000	chr8	86977321	G	A	found	no	yes
LIP	chr9	40934406	chr9	42581656	T	A	found	no	yes
LIP	chr1	10431748	chr1	10544851	G	A	found	no	no
LIP	chr17	29582244	chr17	29483913	G	A	found	yes	yes
LIP	chr26	33187017	chr26	34447304	T	C	found	yes	yes
LIP	chr30	4510951	chr30	4795900	C	T	found	yes	yes
LIP	chr4	47113988	chr4	47285204	A	T	found	yes	yes
LIP	chr4	95252068	chr4	95364574	A	T	found	yes	yes
LIP	chr9	3085222	chr9	3118093	C	A	found	yes	yes
LIP	chr1	122283468	chr1	123426208	A	C	found	yes	no
LIP	chr14	52250726	chr14	51556138	C	T	found	yes	no
LIP	chr14	53324326	chr14	52653121	A	G	found	yes	no
LIP	chr17	16573635	chr17	16642725	T	C	found	yes	no
LIP	chr18	15530280	chr18	15595862	T	A	found	yes	no
LIP	chr2	4044657	chr2	4059259	A	C	found	yes	no
LIP	chr2	76393138	chr2	76471715	G	C	found	yes	no
LIP	chr2	107860807	chr2	108203154	A	C	found	yes	no
LIP	chr20	29358869	chr20	30270730	C	T	found	yes	no
LIP	chr22	573161	chr22	619278	A	G	found	yes	no
LIP	chr22	23971258	chr22	24676750	A	G	found	yes	no
LIP	chr22	23971258	NW_019646 087.1	8781	A	G	not found	yes	yes
LIP	chr26	40048597	chr26	41317337	A	T	found	yes	no
LIP	chr3	81356650	chr3	83166511	G	A	found	yes	no
LIP	chr31	2013281	chr31	8651746	T	C	found	yes	no
LIP	chr4	9684807	chr4	9719703	C	G	found	yes	no
LIP	chr5	30402947	chr5	27642878	A	C	found	yes	no
LIP	chr7	44303415	chr7	45561401	A	C	found	yes	no
LIP	chr7	77372524	chr7	79547823	T	G	found	yes	no
LIP	chr14	296600	NW_019643 582.1	163072	T	G	not found	no	yes

LIP	chr18	56233143	chr18	56332502	C	T	not found	no	yes
LIP	chr2	13626858	chr2	13676181	G	C	not found	no	yes
LIP	chr1	115504590	NW_019646 122.1	28415	T	C	not found	no	no
LIP	chr1	115504590	chr1	116526282	T	C	not found	no	no
LIP	chr1	115504590	chr1	116643212	T	C	not found	no	no
LIP	chr15	18516837	NW_019643 418.1	116109	T	G	not found	no	no
LIP	chr15	18516837	NW_019643 394.1	211146	T	G	not found	no	no
LIP	chr15	18516837	chr15	19197504	T	G	not found	no	no
LIP	chr18	9517699	chr18	9673823	G	C	not found	no	no
LIP	chr2	86268	chr2	80336	A	C	not found	no	no
LIP	chr2	26636712	chr2	26706669	G	A	not found	yes	yes
LIP	chr21	1082137	chr21	133290	G	T	not found	yes	no
LIP	chr7	53003749	chr7	55021516	A	G	not found	yes	no
LIP	chr7	53003761	chr7	55021528	G	T	not found	yes	no
LIP	chr8	1656435	chr8	3462742	C	T	not found	yes	no
LIP	chr8	4013962	chr8	6017687	A	C	not found	yes	no
LiP	chr1	113086506	chr1	114032630	C	G	just in EquCab3	no	no
LiP	chr1	163121631	chr1	165088722	C	T	just in EquCab3	no	no
LiP	chr11	60729384	chr11	61092938	C	G	just in EquCab3	yes	no
LiP	chr13	12281767	chr13	12591780	T	A	just in EquCab3	yes	no
LiP	chr14	53281174	chr14	52609933	G	T	just in EquCab3	yes	no
LiP	noalign	noalign	chr15	14765848	C	T	just in EquCab3	no	no
LiP	noalign	noalign	chr16	40321052	C	G	just in EquCab3	no	no
LiP	noalign	noalign	chr16	40321063	C	T	just in EquCab3	no	no
LiP	chr18	98589	chr17	112172	T	C	just in EquCab3	yes	no

LiP	chr17	22409571	chr17	22310438	A	G	just in EquCab3	no	no
LiP	chr2	63488928	chr2	63948251	C	T	just in EquCab3	yes	no
LiP	chr20	30303426	chr20	31215223	A	G	just in EquCab3	no	no
LiP	chr20	41448335	chr20	42348483	C	G	just in EquCab3	no	no
LiP	scaffold_8_8	538547	chr21	762973	T	C	just in EquCab3	yes	no
LiP	scaffold_1_90	742	chr21	762973	T	C	just in EquCab3	yes	no
LiP	chr21	1345122	chr21	1955710	G	A	just in EquCab3	yes	no
LiP	chr21	46651305	chr21	47934770	T	C	just in EquCab3	no	no
LiP	scaffold_1_17	54314	chr24	207208	C	G	just in EquCab3	yes	no
LiP	scaffold_1_17	54302	chr24	207220	G	C	just in EquCab3	yes	no
LiP	chr24	3434129	chr24	3299008	T	C	just in EquCab3	no	no
LiP	chr26	39072509	chr26	40344282	T	C	just in EquCab3	yes	no
LiP	scaffold_1_80	61745	chr29	552846	C	T	just in EquCab3	yes	no
LiP	chr3	85720365	chr3	87844985	G	A	just in EquCab3	no	no
LiP	chr4	89013190	chr4	89127688	A	G	just in EquCab3	yes	no
LiP	chr5	6347982	chr5	6363111	G	A	just in EquCab3	no	no
LiP	chr5	49245871	chr5	45822661	G	C	just in EquCab3	yes	no
LiP	noalign	noalign	chr6	28015944	G	T	just in EquCab3	yes	no
LiP	chr8	82614	chr8	1810043	A	T	just in EquCab3	yes	no
LiP	scaffold_1_87	8272	chr8	1897212	G	A	just in EquCab3	no	no
LiP	scaffold_1_03	55207	chr8	1990358	G	C	just in EquCab3	yes	no

LiP	scaffold_1 07	177061	chr8	1990358	G	C	just in EquCab3	yes	no
LiP	chr8	1768323	chr8	3580793	C	T	just in EquCab3	yes	no
LiP	chr8	2072691	chr8	3580793	C	T	just in EquCab3	yes	no
LiP	chr8	2177024	chr8	4179490	T	C	just in EquCab3	yes	no
NOR	chr11	7649446	chr11	7638666	C	T	found	no	yes
NOR	chr17	20005831	chr17	19909100	C	T	found	no	yes
NOR	chr18	21015205	chr18	21081929	C	G	found	no	yes
NOR	chr8	31590451	chr8	34654749	C	G	found	no	yes
NOR	chr23	8657794	chr23	8072201	A	G	found	no	no
NOR	chr1	115297935	chr1	116319664	C	T	found	yes	yes
NOR	chr10	21323697	chr10	21613706	C	A	found	yes	yes
NOR	chr11	38548898	chr11	38843410	C	T	found	yes	yes
NOR	chr17	4565574	chr17	4636020	G	C	found	yes	yes
NOR	chr29	1231244	chr29	2241954	C	A	found	yes	yes
NOR	chr7	64671737	chr7	66853582	T	C	found	yes	yes
NOR	chr11	41767183	chr11	42088312	C	T	found	yes	no
NOR	chr16	2803472	chr16	4274662	G	C	found	yes	no
NOR	chr19	36357963	chr19	38941787	G	A	found	yes	no
NOR	chr19	36796471	chr19	39380133	A	T	found	yes	no
NOR	chr19	58455252	chr19	61335678	C	T	found	yes	no
NOR	chr20	1889151	chr20	2144556	C	T	found	yes	no
NOR	chr20	24456926	chr20	25180104	A	G	found	yes	no
NOR	chr21	1018747	chr21	204090	C	G	found	yes	no
NOR	chr21	2605508	chr21	3228070	A	T	found	yes	no
NOR	chr21	31429891	chr21	32453825	T	A	found	yes	no
NOR	chr22	43893158	chr22	44860978	C	T	found	yes	no
NOR	chr31	16751314	chr31	16791438	A	G	found	yes	no
NOR	chr8	3702372	chr8	5726905	G	T	found	yes	no

NOR	chr8	4890346	chr8	7004112	A	T	found	yes	no
NOR	chr8	4890346	chr8	5851711	A	T	not found	no	no
NOR	chr8	11081101	chr8	13296362	G	A	found	yes	no
NOR	chr8	11081113	chr8	13296374	C	T	found	yes	no
NOR	chr23	199384	NW_019644 111.1	13075	C	G	not found	no	no
NOR	chr23	252882	NW_019644 035.1	7983	C	T	not found	no	no
NOR	chr14	473741	NW_019643 582.1	34819	T	A	not found	yes	no
NOR	chr27	596466	NW_019645 822.1	25127	T	G	not found	no	no
NOR	chr27	596466	NW_019643 525.1	46174	T	G	not found	no	no
NOR	chr27	596466	chr27	598908	T	G	not found	no	no
NOR	chr8	1799905	chr8	3612240	C	T	not found	no	yes
NOR	chr8	2565803	chr8	4567587	T	G	not found	no	no
NOR	chr6	3270286	chr6	2944071	T	C	not found	no	yes
NOR	chr23	7864535	NW_019643 688.1	274190	G	A	not found	yes	no
NOR	chr14	9869853	chr14	9098133	A	C	not found	no	no
NOR	chr12	12435069	chr26	15321210	C	T	not found	no	no
NOR	chr10	13100245	chr10	13279495	G	A	not found	yes	no
NOR	chr10	13100245	chr10	13110832	G	A	not found	yes	no
NOR	chr10	14126430	NW_019644 715.1	18830	A	T	not found	yes	no
NOR	chr10	14126430	chr10	14310057	A	T	not found	yes	no
NOR	chr10	14151126	chr10	14333736	G	T	not found	yes	yes
NOR	chr12	17455667	chr12	20972468	C	T	not found	no	yes
NOR	chr12	17467671	chr12	20984465	C	G	not found	no	yes
NOR	chr28	20549421	chr28	21592630	C	T	not found	no	no
NOR	chr12	22821184	chr12	26369633	C	T	not found	no	no
NOR	chr10	23731364	chr10	24166815	T	C	not found	no	yes
NOR	chr11	25089146	chr11	25163875	C	A	not found	no	

NOR	chr20	32276595	NW_019645 272.1	15516	T	C	not found	no	yes
NOR	chr20	32276595	NW_019644 819.1	52034	T	C	not found	no	no
NOR	chr20	32276595	chr20	33276510	T	C	not found	no	no
NOR	chr20	32276595	chr20	33139015	T	C	not found	no	no
NOR	chr22	36838245	chr22	37737690	T	C	not found	yes	no
NOR	chr23	40878726	chr23	40553043	G	A	not found	no	yes
NOR	chr23	40878771	chr23	40553088	C	T	not found	no	no
NOR	chr11	41656509	chr11	41981138	T	A	not found	no	no
NOR	chr7	43560898	chr7	44599012	T	C	not found	yes	yes
NOR	chr1	44584336	chr1	44944788	G	A	not found	yes	no
NOR	chr1	44584336	chr1	44913757	G	A	not found	yes	no
NOR	chr8	49612506	chr8	52686429	G	T	not found	no	yes
NOR	chr16	65435532	chr16	67018600	T	C	not found	no	no
NOR	chr3	97439027	chr3	99255299	A	G	not found	no	no
NOR	chr1	155138900	NW_019646 251.1	75746	G	C	not found	no	yes
NOR	chr1	155138900	NW_019643 074.1	75753	G	C	not found	no	no
NOR	chr1	155138915	NW_019646 251.1	75761	T	C	not found	no	no
NOR	chr1	155138915	NW_019643 074.1	75768	T	C	not found	no	no
NOR	chr1	114280422	chr1	115300895	A	C	just in EquCab3	yes	no
NOR	chr1	114234815	chr1	115300895	A	C	just in EquCab3	yes	no
NOR	chr1	157856346	chr1	159473532	A	G	just in EquCab3	yes	no
NOR	chr11	41636525	chr11	41934046	C	T	just in EquCab3	yes	no
NOR	chr12	12410783	chr12	12632000	A	G	just in EquCab3	yes	no
NOR	chr12	17467709	chr12	20984503	C	T	just in EquCab3	no	no

NOR	chr12	17368623	chr12	20984503	C	T	just in EquCab3	no	no
NOR	chr12	17356600	chr12	21028811	C	T	just in EquCab3	no	no
NOR	chr12	17356726	chr12	21028937	T	A	just in EquCab3	no	no
NOR	chr12	32767000	chr12	35488348	C	T	just in EquCab3	yes	no
NOR	chr13	8283509	chr13	8571371	A	G	just in EquCab3	no	no
NOR	scaffold_2_94	47503	chr13	9212502	C	T	just in EquCab3	yes	no
NOR	chr14	963402	chr14	209691	C	T	just in EquCab3	yes	no
NOR	chr14	9078851	chr14	8307528	A	C	just in EquCab3	no	no
NOR	chr14	92099865	chr14	92754738	T	A	just in EquCab3	no	no
NOR	scaffold_3_40	31703	chr15	35289	T	C	just in EquCab3	yes	no
NOR	chr18	71216313	chr18	71333930	C	T	just in EquCab3	no	no
NOR	scaffold_1_31	79348	chr21	173964	G	A	just in EquCab3	yes	no
NOR	chr24	294298	chr24	153809	T	A	just in EquCab3	yes	no
NOR	chr24	38138711	chr24	38509801	G	A	just in EquCab3	no	no
NOR	chr26	227587	chr26	617551	A	G	just in EquCab3	no	no
NOR	scaffold_11_1	37009	chr27	568230	A	G	just in EquCab3	yes	no
NOR	chr28	35964604	chr28	37095887	G	A	just in EquCab3	yes	no
NOR	chr28	35990989	chr28	37113509	T	C	just in EquCab3	yes	no
NOR	scaffold_9_3	491312	chr29	891655	A	G	just in EquCab3	yes	no
NOR	chr14	565989	chr29	1875093	C	T	just in EquCab3	no	no
NOR	chr29	770364	chr29	1875093	C	T	just in EquCab3	no	no

HAF	chr10	59172497	chr10	60310985	G	A	called	no	yes
HAF	chr17	1685198	chr17	1753762	T	C	called	no	yes
HAF	chr2	100761774	chr2	101097784	G	T	called	no	yes
HAF	chr3	53100714	chr3	54576398	C	A	called	no	yes
HAF	chr4	44983704	chr4	45153777	G	A	called	no	yes
HAF	chr5	21907765	chr5	19148516	A	G	called	no	yes
HAF	chr5	53320088	chr5	49896738	T	C	called	no	yes
HAF	chr7	91483698	chr7	93696413	T	G	called	no	yes
HAF	chr14	4822552	chr14	4058364	C	T	called	no	no
HAF	chr22	27863665	chr22	28768316	T	C	called	no	no
HAF	chr5	16057269	chr19	3035926	T	A	called	no	no
HAF	chr6	79058078	chr6	80225422	T	C	called	no	no
HAF	chr8	32485953	chr8	35525782	C	T	called	no	no
HAF	chr1	120334435	chr1	121468596	T	C	called	yes	yes
HAF	chr12	13784646	chr12	14498413	T	A	called	yes	yes
HAF	chr14	4827882	chr14	4063623	T	C	called	yes	yes
HAF	chr16	2815931	chr16	4288452	T	C	called	yes	yes
HAF	chr20	29311733	chr20	30220575	C	T	called	yes	yes
HAF	chr12	13825556	chr12	14539782	G	A	called	yes	no
HAF	chr13	19811302	chr13	20890984	A	G	called	yes	no
HAF	chr14	4827896	chr14	4063637	A	C	called	yes	no
HAF	chr17	39695905	chr17	39599876	T	C	called	yes	no
HAF	chr18	4152059	chr18	4122138	A	G	called	yes	no
HAF	chr20	26396785	chr20	27301180	A	T	called	yes	no
HAF	chr21	49759250	chr21	51009814	C	G	called	yes	no
HAF	chr22	552168	chr22	598289	G	A	called	yes	no
HAF	chr31	16997011	chr31	17040610	T	C	called	yes	no
HAF	chr6	13857585	chr6	13645648	T	C	called	yes	no
HAF	chr6	38444589	chr6	39579282	T	G	called	yes	no

HAF	chr6	38444606	chr6	39579299	A	G	called	yes	no
HAF	chr7	9200806	chr7	9677163	A	G	called	yes	no
HAF	chr25	4135	NW_019642 813.1	331601	G	A	not called	no	no
HAF	chr18	52005	chr18	65931	C	T	not called	no	yes
HAF	chr18	52005	chr17	47646	C	T	not called	no	no
HAF	chr24	540464	chr24	405239	A	G	not called	yes	yes
HAF	chr11	1039845	chr11	1041777	G	C	not called	no	no
HAF	chr19	4938297	chr19	7325062	C	A	not called	yes	no
HAF	chr10	7931916	chr10	8047442	G	A	not called	no	yes
HAF	chr23	8139725	NW_019643 688.1	553562	C	T	not called	yes	no
HAF	chr18	9582002	chr18	9033716	A	G	not called	no	no
HAF	chr18	9582002	chr18	12909306	A	G	not called	no	no
HAF	chr12	14048779	chr12	14953906	C	T	not called	no	no
HAF	chr12	14048779	chr12	14934992	C	T	not called	no	no
HAF	chr12	14048779	chr12	14879021	C	T	not called	yes	no
HAF	chr12	15029981	NW_019642 075.1	5881	T	C	not called	no	no
HAF	chr13	19315749	chr13	20396513	C	T	not called	yes	no
HAF	chr22	24976358	chr22	25875039	G	A	not called	no	no
HAF	chr22	24976376	chr22	25875057	C	T	not called	yes	yes
HAF	chr26	32427893	chr26	33689194	T	C	not called	no	no
HAF	chr26	32427893	chr26	33752767	T	C	not called	no	no
HAF	chr26	32427893	chr26	33673583	T	C	not called	no	no
HAF	chr7	34653199	chr7	35818941	A	G	not called	yes	no
HAF	chr7	34653210	chr7	35818952	A	G	not called	yes	no
HAF	chr2	42919845	chr2	43141745	G	T	not called	no	no
HAF	chr5	48921158	chr5	45470619	T	C	not called	no	no
HAF	chr3	54907512	chr3	56379955	C	T	not called	no	yes

HAF	chr6	58762174	NW_019642 687.1	11347	C	G	not called	no	no
HAF	chr7	59514418	chr7	61694002	T	C	not called	no	no
HAF	chr11	60756515	chr11	61120069	G	T	not called	no	no
HAF	chr18	65542248	chr18	65657094	A	G	not called	no	no
HAF	chr6	78694433	chr6	79861978	C	T	not called	yes	no
HAF	chr14	92829544	chr14	93591845	C	A	not called	no	no
HAF	chr14	92829544	chr14	93486571	C	A	not called	yes	no
HAF	chr14	92870205	chr14	93560815	G	A	not called	no	yes
HAF	chr1	143424677	chr1	144891989	C	A	not called	no	yes
HAF	chr1	162587371	chr1	164688749	T	C	not called	no	no
HAF	chr1	163046056	chr1	165012407	G	A	just in EquCab3	no	no
HAF	chr11	52232188	chr11	52582710	A	G	just in EquCab3	yes	no
HAF	chr11	52232228	chr11	52582750	C	T	just in EquCab3	no	no
HAF	chr11	60729070	chr11	61092624	C	A	just in EquCab3	yes	no
HAF	chr12	31057784	chr12	34882799	T	C	just in EquCab3	yes	no
HAF	chr13	18159913	chr13	19237651	A	C	just in EquCab3	yes	no
HAF	chr13	18160181	chr13	19237678	A	G	just in EquCab3	yes	no
HAF	chr13	21927948	chr13	23558006	A	C	just in EquCab3	yes	no
HAF	chr13	26958570	chr13	26404373	G	A	just in EquCab3	yes	no
HAF	chr18	13036153	chr18	12929475	G	A	just in EquCab3	yes	no
HAF	chr2	34726954	chr2	34906791	G	C	just in EquCab3	no	no
HAF	chr20	31048188	chr20	31912373	G	T	just in EquCab3	yes	no
HAF	scaffold_2 24	5223	chr20	51466011	T	G	just in EquCab3	yes	no

HAF	scaffold_8_8	561709	chr21	788265	G	A	just in EquCab3	yes	no
HAF	scaffold_1_11	31059	chr24	51881	T	A	just in EquCab3	no	no
HAF	chr14	673521	chr24	291480	A	T	just in EquCab3	no	no
HAF	chr24	455123	chr24	314661	A	C	just in EquCab3	no	no
HAF	scaffold_8_5	1123414	chr27	26964	C	T	just in EquCab3	yes	no
HAF	scaffold_1_80	27440	chr27	26964	C	T	just in EquCab3	yes	no
HAF	scaffold_1_96	74869	chr27	79930	C	A	just in EquCab3	yes	no
HAF	noalign	noalign	chr28	18454	A	G	just in EquCab3	yes	no
HAF	chr6	38004787	chr6	39063395	T	A	just in EquCab3	yes	no
HAF	chr6	38004798	chr6	39063406	A	G	just in EquCab3	yes	no
HAF	scaffold_8_3	413845	chr6	86814340	A	G	just in EquCab3	yes	no
HAF	chr8	70163030	chr8	73645663	A	C	just in EquCab3	no	no
HAF	chr9	38880815	chr9	40536852	C	T	just in EquCab3	yes	no

Table A.C.2: Detailed information of ‘de novo’ candidates obtained from GATK’s PhaseByTransmission (PBT) algorithm in Lipizzaner trio. 111, 113 and 166 were the sample IDs of Lipizzaner son, father, and mother respectively.

TRIO	EQ2 chr	EQ2 pos	EQ3 chr	EQ3 pos	Ref	Alt	Depth (111/ 113/ 166)	Found in EQ2 finalized candidate list of Lip	Found in EQ2 finalized candidate list of Lip	Found in Jagannathan’s variations
LIP	chr12	14923621	chr26	15298236	T	C	75/135/142	not found	not found	not found
LIP	chr12	30138931	chr12	33942154	C	A	42/37/31	not found	not found	found
LIP	chr26	14842365	chr26	15341895	A	C	45/83/73	not found	not found	found
LIP	chr26	14842365	chr12	12626648	A	C	45/83/73	not found	not found	not found

LIP	chr31	16520917	chr31	16561393	T	C	453/368/326	not found	not found	found
LIP	chr7	98533779	NW_019643487.1	4355	G	A	29/62/93	not found	not found	not found

Table A.C.3: Detailed information of variants which were validated in the laboratory. Primer sequences and sequences produced by Sanger resequencing were seen.

TRIO	EQ2 chr	EQ2 pos	Sanger result	Category	Variant in brackets including flanking region	Primer forward ID	Primer forward seq.	Primer reverse ID	Primer reverse seq.	amplicon length (bp)
LIP	chr1	15859530	SNP validated	True denovo variant	ataccctgttccccattactaa catcttacattagatggtacattt gttacaattaatgaccagattg atccattattagtaactaaagtc atagcttattcagattcccccttagtt ttacctaattgccctttctgtttt gggatccatccaggacacca cattagatttagttgtagtctcc ttaggctcctcttggtgtgaca gtttccaagactcccctgtttttg atgacctgacagttttgaggag tctgttatttgaggactatatt gtaggatgacc[c/g]tctcttg gaattgtctgatgttttctcagg atttgactgaggttatgggtttg ggaaagatcacagaggcaaacg accatttgcacacatcgtatca aaggcaccctgctatcagctgat ctatgattgtgatgtgatctttat cgcttgctgaagtgtgtgtgt caagtctcctcactgcagagtta cttttctcctccttccatact gtactcctggaagaatactac acatagcctactcctaaggagt aggggtgggggttatgctcca tctcattgaggg	chr1:15859420.fwd	CTCCTT AGGCTC CTCTTG GC	chr1:15859705.rev	TTGAC CAACA CCACT TCAGC	286
LIP	chr1	15864504	SNP validated	True denovo variant	atagttaatgatgttgaacatcttt tcattgctcctgttggccatctgta tatttctttggagaaatctgtt caggtctttgcccagtttttaatt tgggtgtgtgttttctgtgtgga ttgtatgagttctgtatatttg actcttaacccttatcaatata ggtttgcaaatatctctctctatt gttaggctgtcttttgtttgtga tggttcctttgctgtagaagc ttttactttgggtgtagaccaatt gtttattttctattgttccctt[g/ t]cctggtcagacagggtacttg aaaatatgctgctaagactaatg tcaaagagcgtactgcctatgt ttctctagaagttttatggtttca ggtcttacattcaaatcttaatcc atttgagttattttgtataggt gtaagataatgctccacttcatt ctttgcatgtgctgccagtttt cccaacaccatgtattgaagag actttcctttccattgtacctct taactcttctcaaaaatagct gtccatgtctgcccgtagc caagtcttaagtt	chr1:15864504.fwd	aatatctgttc aggctctttg c	chr1:15864504.rev	ctacggg gccagca catg	524

LIP	chr1	68664504	SNP validated	True_deno vo variant	tgaagattggctcaaatgttag ctcagggtgtcaatcttaaaaaa aataaaatagaattaagttttat tcaaatatggttttattagagataa tttacttggcatcaaacattgca taaagcatacgtgaggttaactt ttaatgataatacaaacacagt cagcccagatcttctagacttt gtaatactttgtaatactagcatt aaagatagtaataaggtgtcttt gaaaaagctgttccataaccaa atatttgggaacactgagtaaa acaaagttaaatacatta[a/c] aaaaatcaagactccctctga gattcaaatattaattgtaataat tattgtattagttatctattgctat gtaacaaattgtttaaatagtg gctaaaaacaagaataatg gaatgctgttacagtctctgtgg gtcaggaaattgtgagcagcttg gttgagggtctctgctcagggt tctctcacagaggacagctcca gctgttggtggggaagcccc atctgaaagcttgactggggt ggaggaaaccactccaagatg cctcactcactggtggcaagt tg	chr1:686 64364fw d	ACAGTC AGCCCA GATCTT CC	chr1:68 664677r ev	CTCAA CCAAG CTGCT CACAA	314
LIP	chr1 0	82141638	SNP validated	True_deno vo variant	acaattctaagataaaggcattc ctatgcaaacctgtaattgtaa aatgaaagacagaaatagcaa aagtggtcacagcacaatagt gtacattacagcatatttggct ttatatacacttgaatttccat aatttaaatgagttaaactcaa atgaaaaatgacattttctgaat actgaggatcacgtccctgtta agagctctatttttaaacagagt aagcagctctaatgaaacaa aacccaaataagcatttaactta actagtggctcaaatagttac[g/a]tctgtggaacaagaaaa aaattccaactgcatcacgttg gtgattaaaaagcattttatcgc tccagaaggtacaggtgtttctc aattctaaaatgaaacagcac gtgccagactgctgatafgca aaagtgtctgatcaaggaaac acaggattatagacttgactc acataagctcaaacgtctgtg ctgttcagaggcaaatagaag gtcccaataaaaaggttcagg agaagaaaagaagtcacacactt tctccctagcaattcacatcaa gatagggaat	chr10:82 141528f wd	AGGATC ACGTGC CCTGTT AA	chr10:8 2141853 rev	TGCCT CTGAA CAAG ACAA G	326
LIP	chr1 3	27768579	SNP validated	True_deno vo variant	agaagacattggactctctgg ggctcattttctcatctgtaaaa tgggctatgatagttctcact cactgggctactgaaagatta aatgggttaaccacataaagc atacagaaaagaccgcctctag aaaaaacctgctcaatgtggctt cattatcatcaccacatcat cctgtgtgtaactctgacacag atgttatatttctaatgtaaatc ctaaaaataaatgactaggcc aaaagatatgattttgatatag acagctctgatcagcaatggaga [t/v]tattataaattgacaagtt ggtaagttgtttctctcggg gacccccgtgagtgctctgtg gtctggagacacatttaagatc aggttcatccactgctccccg cttctgttcattagcagatg ctctgaacggtcccagcctg	chr13:27 768420f wd	ACTGCT CAATGT GGCTTC AT	chr13:2 7768809 rev	CATTG CTCCT TCCGT TCCTT	390


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ttttactctctacgttatattcac
caccctagacttcttaaagatcc
cgcaaggaaacggaagga
gcaatgcaggtggcgtggat
gtgagggtctgctccggcgt
gtcctggacagagcgacctcc
gaggaaagcaca

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LIP	chr1 9	43987271	SNP validated	True_deno vo variant	ggttattctctgtgaatgcgact taggctgctttttctccctage cagctgttcacccttcattatctt tgtctctctattttcacagttc tttttaagattggacctgagc taacaactgtgtaacttttttt tttttagctttttccccaatccc cccagaacatagttgtatattta gtgtgggtcctctagttgtggg atgtgggacaccgctcaaat ggcctaaccgagcagtgccatgt cgcacccagatccgaacc tggccgccaaag[c/t]ggaa tgcgacacttagcactcggc cacaggccagccctgtttc agttcttctcttttatctccc taaatgccctctctgtttttct cttctctgattttgtcctcagtc tcttaccagtggtctggaact attgagtattccagaccagctga aggggtgattgtgctgctcag gcctgaggcaggaatcca tctgtttggagcctggacct ctgtggcccaagccattgtctt ggcttttctagtaatgagatgg gagggtgcctcc	chr19:43 987179f wd	CTTCTA GTTGTG GGATGT GGG	chr19:4 3987567 rev	GCAGC CCTCC CATCT CATT	389
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LIP	chr2	49679736	no SNP		gacaaacctttagctagagtc cgaagaaaaaagagagaag gctcaataaataaatacaaaa atgaaagaggagagattacaa gggacacctcagaatgcaaa agatgataagagaatactatga aaagctatacgcaaaaattg gataatagaaatggata aattctagaacatacaaccttc caaaaactggccaagaagatgt agaaaattgaaatgaccaatca ccagttaagagatcaaaacag caatcaaaaactcccaaaaa caaaagtcaggcca[g/t]a tggcttccctggtgaattctacc aaacattcaagaagacttaata cctatcttcaaaacttccaa aagattgaagagaggggag gcttcttaactattctacgaag ccaagatcttgatacaaaa ccagacaaggacacacaaaa aaaacaaaattacaggccaata tcaactgaacatcgatgcaaaaat cctcaacaaaactagcaaat cgaatacgataatacattaaaaa gatcatacatgatcaagcg ggtttcattccaggatgcagg gatggttc	chr2:496 97936.f	aggagagat tacaaggga cacc	chr2:49 697936. r	cctggaat gaaacc gcttg	516
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LIP	chr2	69806759	SNP validated	True_deno vo variant	accatctgatgactctttttctc tgtgatgcaagagacaagccat ctgcaaaaatgaagataagg gtctgaaattaaagtgagtaga aaatgtagaaacagttattgtg gaatgcagtcagcaagctga ccacgaaatgtagcaggtttg ctggacagtgaaggctaactg agattggaggttaataattaaa agtgttccaactgacttcttc gtttggccaggaacataatg	chr2:698 06611fw d	GTTTGC TGGACA GTGAAG GG	chr2:69 806944r ev	ATGTC GGAC ACCTA CCACA G	334
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tgcctggatgaagacttcagga
 ttcttccagaacaatgagatgaa
 gacagag[a/c]agcaaggaa
 atctgtaatttgacagcaatfat
 gaaataaagaattagagaatc
 tggggccagccgggtggtgca
 gcggttatgtgcatgtlccgc
 atcagtgccctggggttcgccg
 gtttggatcccgggtgggacg
 tagcaccctggcagccatg
 ctgtgtaggtgtccgacatata
 aagtagaggaagatgggacag
 gatgttagctcaggccagctct
 cctcagcaaaaagaggaagatt
 ggacagtagtttagggcta
 atcatcctaaaacaaaaaaaaa
 ag

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LIP	chr9	3085222	SNP also in parent	ttgactctgaattgtttatgacattataagaatcgccatgga ccagttaattagctcaactagttc ttcaacaacatacgtctaaagct attcaacatacaaaattcttgcattatthaatatgttgaagtcactc cagtatttcccagaagtcata gaattatatagtatattgcatgct gtgtatgctgtatacatacatgt atgtatataatgaatgtgtgttt atgcatgaagatatttactgtgtg tgcacataatataataacttata catacgtat[c/a]aaaacataa aatacatacataaaaagtttaa aataacttacagaacaaaaa aagtcagccaatgctgtttaa ggcaaaactgaatccagatg cccaagccttagcattcaca ctctccagctagaagaatcctat gtcgaacaatgaagggtcca cagcactgtgtgtaattaaggc aaaggaggcagaactcttct actgttgatgcatgcatgctt tctttacagcaagccttttcaa caactgtcccttagctcatattc agattgccacctaggag	chr9:308 5222.f	gccatggac cagtaatta gct	chr9:30 85222.r	gctggag agtgtgaa tgtgc	393
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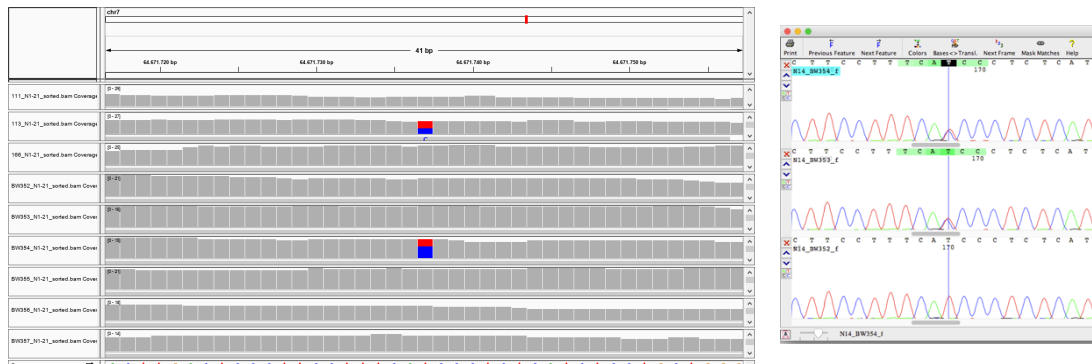


Figure A.D.2: IGV screenshot and Sanger result of a ‘de novo’ candidate (chr7:64671737) which was also detected in one of the parents of BW-354 in the top and left respectively. However, it seemed like parents do not carry the mutation in the IGV screenshot, Sanger result detected the mutation in BW-353 (Noriker mother).



Figure A.D.3: IGV screenshot and Sanger result of a ‘de novo’ candidate (chr4:44983704) that called as no SNP depending on the validation result in BW-357. However, the candidate seems like a ‘de novo’ mutation, Sanger sequencing did not detect any variant at the position.

APPENDIX E

LOGARITHMIC VERSION OF PSMC

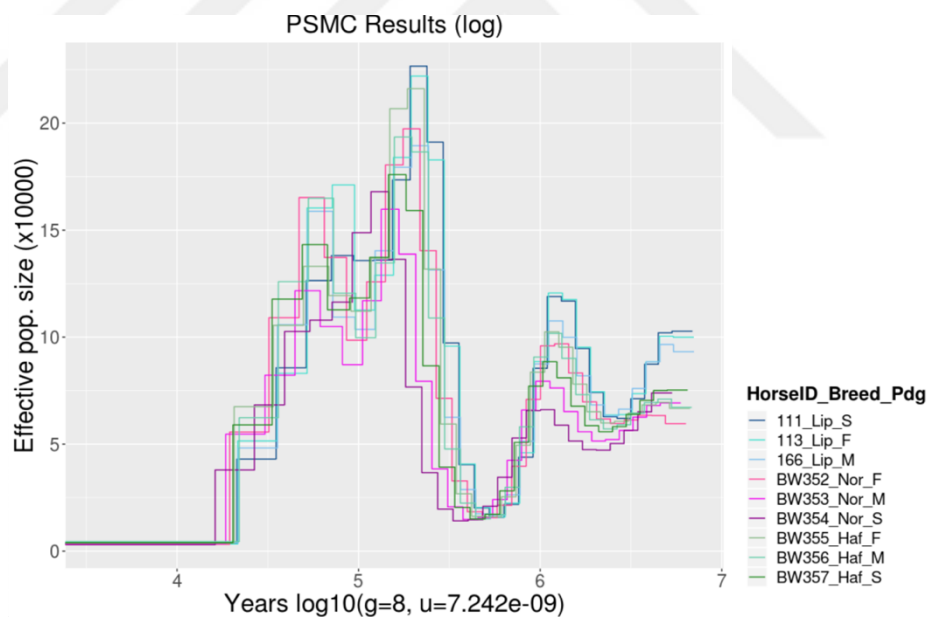


Figure A.E.1: Logarithmic version of PSMC graph. All horses showed similar patterns in the graph, but 111 and 113 had a larger effective population size than the other trios during the timeline.