SEQUENCE-BASED ANALYSES OF THE GOETTINGEN MINIPIG GENOME

Dissertation

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SUMMARY

Among the known pig breeds, the Göttingen Miniature Pig (GMP) stands out due to its special characteristics and history. Its current appearance dates back to the 1960´s when animal scientists from Goettingen took the effort to breed a particularly small, white-coated pig for laboratory use. For this purpose, a total of three breeds with different characteristics, the Minnesota Minipig, the Vietnamese Potbellied Pig and the German Landrace were crossed until the new breed met the expectations. With a weight of 35 to 45 kg, adult minipigs are considerably smaller than normal sized fattening breeds and pigmentation has almost disappeared. Although the breeding of the GMP has been scientifically accompanied from the beginning, the exact background of the dwarfism is so far unknown.

In recent years, more and more molecular genetic techniques have entered into animal breeding, and so today's Next Generation Sequencing (NGS) allows the entire genome of an individual to be deciphered at an acceptable cost. This technique will now be used in this study to further investigate the genetic background of size of the GMP. In addition, the breeding of a laboratory animal in very few isolated stocks implies that the danger of population stratification, sub-division of the parent population into sub-populations with different characteristics, is omnipresent but not desired. In this regard, on the basis of NGS data we try to identify possible differentiation between the individual breeding stocks and to assess whether breeding countermeasures are necessary.

In **chapters 2** and **3**, we use whole genome sequence data from various large-breed pig breeds to compare them to full-genome sequence data from ten miniature female Goettingen Minipigs and ten MiniLEWE, another miniature pig breed, as well as a DNA pool made up from ten other MiniLEWEs. Assuming that selection for a small size favoured similar genes in the two miniature swine breeds, we searched for regions in the genome where the genetic variability was reduced due to directional selection but at the same time the regions were highly differentiated from the respective regions in large breeds. Depending on the thresholds used for the three statistics "expected heterozygosity" and F_{ST}, as well as the "composite likelihood ratio test" (CLR), which is based on the distribution of allele frequencies, different genome parts were detected: while 15.7% of the autosomal genome were identified in the first approach in **chapter 2** as signatures of selection, these were only 2% in the second section in **chapter 3**, using much sharper limits. Already the first approach showed that the expected heterozygosity and the CLR test complemented each other by identifying different regions in

which various candidate genes for growth, such as *TGFβ* and *DDR2*, could be localized. In the second part in **chapter 3**, however, the more specific approach significantly reduced the number of regions to be examined. Thus, two possible mechanisms could be identified as a basis for short stature: changes in the MAP kinase pathway and a possible insulin resistance. Furthermore, by analyzing genotype data from a cross-breeding experiment between GMP and large pigs, the inheritance of an exceptionally large swept haplotype on chromosome X could be clarified and shown to account for about 3% of body length in the F_2 cross-breds.

In **chapters 4** and **5**, the population structure of today's GMP, which today is bred in one stock each in Relliehausen in Germany, North Rose in the USA, Nisshin in Japan and two stocks in Dalmose in Denmark, is examined in more detail. From each of the five independent breeding stocks a representative sample of 20 animals was taken. The DNA of every ten of these animals was pooled in a "DNA pool" so that a total of ten pools, two per stock, could be re-sequenced. In addition, data from all breeds already used in the first study were added. By estimating the differentiation between stocks, based on the F_{ST} values for each locus, it was shown in **chapter 4** that GMP is clearly differentiated from other breeds. Nevertheless, there are signs of a beginning separation into three groups: Relliehausen, North Rose and a group consisting of the two Danish stocks and Japan. On the basis of the functional annotation of the SNPs it could be shown that this differentiation takes place mainly in genome regions, which probably are not related to the target phenotypes of the GMP. In the extension of these studies in **chapter 5**, a KEGG pathway analysis attempts to better understand complex biological relationships between genes. This analysis has shown that the individual stocks are not differentiated in most the 316 pathways. However, significant differentiation in the pathway "glutamatergic synapse", which could be related to behavioral traits, could be found between a Danish population and the unit in North Rose. When looking at the overall remaining genetic variability, it became clear that the conservation breeding program in Relliehausen has led to this stock today having the greatest genetic diversity and thus to be regarded as the gene reserve of the GMP breed.

Overall, it was shown that the entire genome of the Goettingen miniature pig can be examined in much greater detail using NGS technology than was the case with SNP marker arrays. The possibility of direct analysis of potentially functional variation, including structural variation, appears to be a great benefit. Nevertheless, their use will be limited to relatively small sample sizes for the foreseeable future, due to the high costs compared to SNP arrays.

ZUSAMMENFASSUNG

Unter den bekannten Schweinerassen sticht das Göttinger Miniaturschwein (GMP) aufgrund seiner besonderen Eigenschaften und Historie heraus. Es geht in seiner heutigen Form auf die Bestrebungen Göttinger Tierzüchter in den 1960er Jahren zurück, ein besonders kleines, rein weißes Schwein für den Laboreinsatz zu züchten. Dazu wurden insgesamt drei Rassen mit unterschiedlichsten Eigenschaften, das Minnesota Minischwein, das Vietnamesische Hängebauchschwein und die Deutsche Landrasse gezielt miteinander verpaart, bis die neue Rasse den Erwartungen entsprach. Mit einem Gewicht von 35 bis 45 kg sind adulte Minischweine erheblich kleiner als normale, zur Nahrungsgewinnung eingesetzte Rassen und Pigmentierung kommt nahezu nicht mehr vor. Obwohl die Zucht des GMP seit Anbeginn wissenschaftlich begleitet wird, liegen die genauen Hintergründe der Verzwergung bislang im Unklaren.

In den letzten Jahren haben immer mehr molekulargenetische Techniken in die Tierzucht Einzug gehalten und so ist es heute durch so genanntes „Next-Generation-Sequencing“ (NGS) möglich, das gesamte Genom eines Individuums zu annehmbaren Kosten zu entschlüsseln. Diese Technik wird nun in dieser Studie dazu genutzt, um das Größenwachstum beim GMP genauer zu untersuchen. Darüber hinaus bringt die Zucht eines Labortieres in sehr wenigen isolierten Beständen es mit sich, dass die Gefahr einer Populationsstratifikation, des Auseinanderdriftens der Ausgangsrasse in Unterrassen mit unterschiedlichen Eigenschaften, allgegenwärtig, jedoch nicht gewünscht ist. Diesbezüglich versuchen wir auf der Basis von NGS-Daten, eventuelle Differenzierung zwischen den einzelnen Zuchtbeständen zu finden und einzuschätzen, ob züchterische Gegenmaßnahmen nötig sind.

In **Kapitel 2** und **3** verwenden wir Vollgenomsequenzdaten von verschiedenen Großschweinerassen, um sie mit Vollgenomsequenzdaten von zehn weiblichen Göttinger Miniaturschweinen und zehn MiniLEWE, einer weiteren Miniaturschweinerasse, sowie eines DNA-pools aus zehn MiniLEWE zu vergleichen. In der Annahme, dass Selektion auf eine geringe Größe in den beiden Minischweinerassen ähnliche Gene favorisiert hat, suchten wir dabei nach Regionen im Genom, in denen die genetische Variabilität infolge gerichteter Selektion deutlich vermindert ist, welche sich aber gleichzeitig stark von denen der Großschweine unterscheiden. Abhängig von den verwendeten Schwellenwerten für die drei verwendeten Statistiken „erwartete Heterozygotie“ und F_{ST}, sowie dem auf der Verteilung der Allelfrequenzen basierenden „Composite likelihood ratio test“ (CLR) wurden unterschiedliche Genomanteile detektiert: Während der erste Ansatz in **Kapitel 2** in 15.7 %

des autosomalen Genoms Spuren von Selektion identifizierte, waren dies im zweiten Abschnitt in **Kapitel 3**, durch die Nutzung deutlich schärferer Grenzwerte, nur 2 %. Bereits der erste Ansatz zeigte, dass die erwartete Heterozygotie und der CLR Test sich ergänzten, indem sie unterschiedliche Regionen identifizierten, in denen unter anderem diverse Kandidatengene für Wachstum, zum Beispiel *TGFβ* und *DDR2,* lokalisiert werden konnten. In zweiten Ansatz in **Kapitel 3** konnte durch das spezifischere Vorgehen dagegen die Anzahl der zu untersuchenden Regionen deutlich vermindert werden. So konnten zwei mögliche Mechanismen, zum einen Veränderungen im MAP-Kinase-Weg und eine mögliche Insulinresistenz als Grundlage des Minderwuchses identifiziert werden. Des Weiteren konnte durch die Analyse von Genotyp-Daten aus einem Kreuzungsexperiment zwischen GMP und Großschweinen, die Vererbung eines außergewöhnlich großen Haplotyps auf Chromosom X geklärt und gezeigt werden, dass dieser etwa 3 % der Körperlänge der Minischweine erklärt.

In den **Kapiteln 4** und **5** wird die Populationsstruktur des heutigen GMP näher untersucht, welches in jeweils einem Bestand in Relliehausen in Deutschland, North Rose in den USA, Nisshin in Japan und zwei Beständen in Dalmose in Dänemark, gezüchtet wird. Dazu wurde aus jedem der fünf unabhängigen Zuchtbestände eine möglichst repräsentative Stichprobe von schlussendlich jeweils 20 Tieren genommen. Die DNA von jeweils zehn dieser Tiere wurde in einem „DNA-Pool“ zusammengefasst, sodass insgesamt zehn Pools, zwei je Bestand, sequenziert werden konnten. Zusätzlich wurden alle bereits in der ersten Studie verwendeten Rassen hinzugenommen. Durch Abschätzung der Differenzierung zwischen den Beständen, anhand der F_{ST} Werte für jeden einzelnen Locus, konnte in **Kapitel 4** gezeigt werden, dass das GMP eindeutig von anderen Rassen abzugrenzen ist. Trotzdem finden sich Anzeichen für eine beginnende Auftrennung in drei Gruppen: Relliehausen, North Rose und eine Gruppe bestehend aus den beiden Dänischen Beständen und Japan. Trotzdem konnte auf Basis der funktionellen Annotation der SNPs gezeigt werden, dass diese Ausdifferenzierung vor allem in Genomregionen stattfindet, welche vermutlich nicht in Verbindung mit den Zielmerkmalen stehen. In der Erweiterung dieser Untersuchungen in **Kapitel 5** wird in Form einer KEGG-Pathwayanalyse versucht, komplexe biologische Zusammenhänge zwischen Genen besser zu erfassen. Diese Analyse hat gezeigt, dass die einzelnen Bestände in den 316 untersuchten Pathways nahezu nie voneinander differenziert sind. Jedoch konnte zwischen einem Dänischen Bestand und der Einheit in North Rose signifikante Differenzierung im Pathway „Glutamatergic synapse“ gefunden werden, welcher mit Verhaltensmerkmalen in Verbindung stehen könnte. Bei der Betrachtung der insgesamt verbleibenden genetischen Variabilität

wurde deutlich, dass das Erhaltungszuchtprogramm in Relliehausen dazu geführt hat, dass dieser Bestand heute die größte genetische Diversität aufweist und somit als Genreserve des GMP zu betrachten ist.

Insgesamt zeigte sich, dass das gesamte Genom des Göttinger Miniaturschweins mit Hilfe der NGS-Technologie deutlich detaillierter untersucht werden kann, als dies noch mit SNP-Markerarrays der Fall war. Die Möglichkeit der direkten Analyse potentiell funktioneller Variation, inklusive struktureller Variation, erscheint als großer Gewinn. Trotzdem wird ihr Einsatz auf absehbare Zeit aufgrund der hohen Kosten im Vergleich zu SNP-Arrays, auf relativ geringe Stichprobenumfänge begrenzt bleiben.

CHAPTER 1

General Introduction

A brief history of the Goettingen Minipig

The Goettingen Minipig (GMP) is a relatively young breed with a diverse genetic background (**Figure 1.1**; Simianer and Köhn 2010). Its roots can be traced back to 1949 when efforts were undertaken to establish a breeding program for its ancestor, the Minnesota Minipig, a small-sized laboratory pig (Dettmers 1956). Feral hogs from Alabama, small, black and likely of European descent (*Sus scrofa ferus*), and another feral hog sampled from Santa Catalina, CA, USA, probably of the genus *Sus vittatus*, were used as foundation. In 1951 and 1953, Piney woods pigs from Louisiana were introgressed and eventually in 1957 Ras-n-Lansa pigs, originating from Guam were introduced. The resulting Minnesota Minipig, was highly variable in colour and weight (Dettmers et al. 1965). Prof. Fritz Haring from the Institute of Animal Breeding and Genetics of the Georg-August-University Goettingen, noticed the demand for non-primate model animals in Europe and initiated a program to breed the Goettingen Minipig based on the Minnesota Minipig.

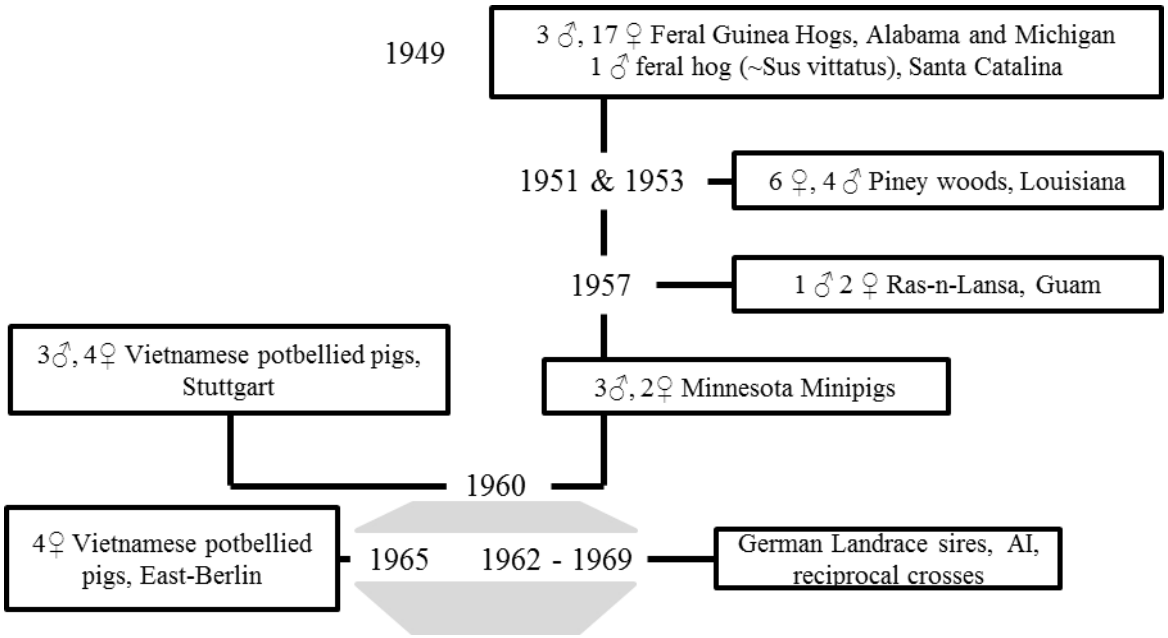


Figure 1.1: Breed history of the Goettingen Minipigs as described in Literature.

Five Minnesota Minipigs were imported in 1960 and mated to seven Vietnamese potbellied pigs (VPP) from Stuttgart zoological gardens, these pigs were dark coated, small and fertile, despite being relatively obese. In 1965, another four VPP individuals were acquired. Albeit exhibiting a wide variety of colours, none of the offspring was uniformly white coated, a trait highly desired by dermatologists. This goal was eventually achieved by introgressing German landrace sires by artificial insemination followed by reciprocal crossing with minipigs and strong selection for small body sizes (Glodek and Oldigs 1981), resulting in a coloured and a

uniformly white coated line. Since then, the pedigree, growth and fertility phenotypes have been completely recorded

The growth of the Goettingen Minipig

When the idea to use miniature pigs in laboratory research came up at the Hormel Institute of University of Minnesota in the 1940's (Dettmers et al. 1965) focus was set on reduction of body size. The intention was to use the pig, due to physiological similarities to humans, but a reduced size would prove advantageous in laboratory and pharmacological testing. This aim was attained, when the GMP was established in the 1960's (Simianer and Köhn 2010) and today the Goettingen Minipig is considered to be the smallest pig breed (**Figure 1.2**) under a controlled breeding scheme (Swindle et al. 2012).

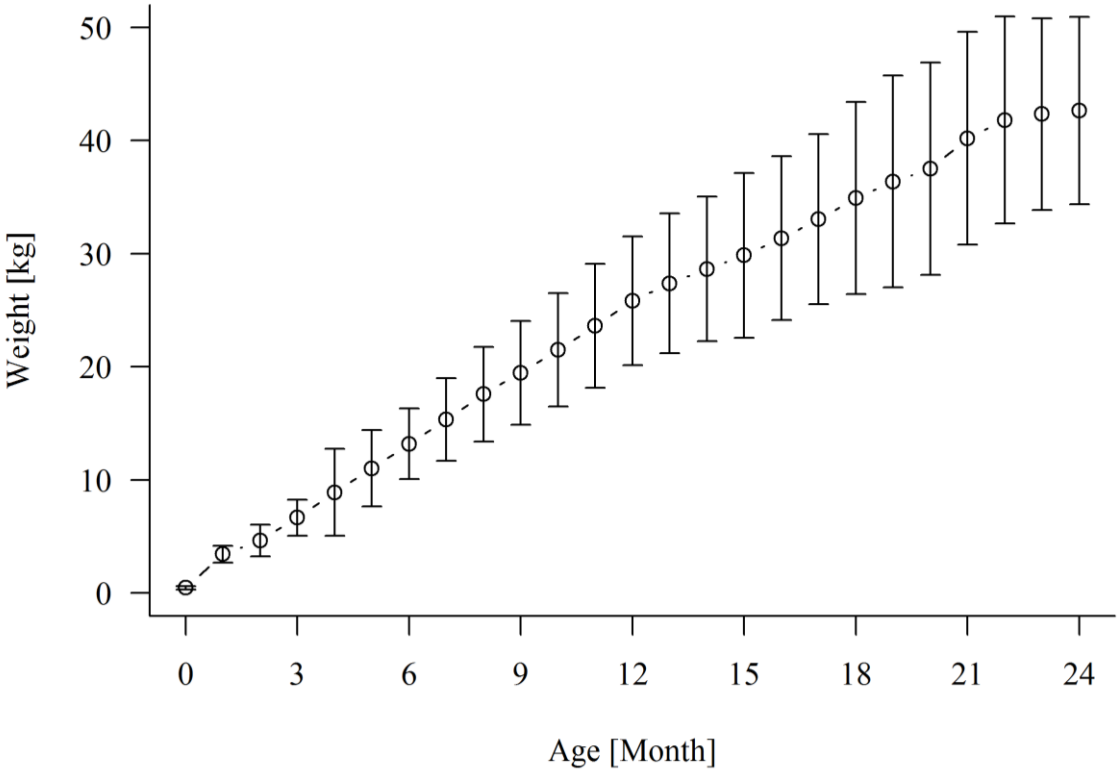


Figure 1.2: Growth curve from all non-pregnant GMP from Denmark. Estimated mean and standard deviation based on recording in 2015-2017.

The dwarfism of the GMP is characterized by a proportional miniaturization (Simianer and Köhn 2010), and does not involve features such as achondroplasia. Since some of the ancestral breeds, selected for their small size, originate from islands, this type is deemed to be a form of insular dwarfism (Simianer and Köhn 2010), a mechanism that generally leads to diminishment of body size of island based mammals and other isolated species, and is thought

to have evolved to cope with restricted nutrition in isolated habitats (Lomolino 2005). Albeit not on an island, pygmies were exposed to similar conditions in rainforest, possibly leading to short stature (Perry and Dominy 2009). Their physiological background is supposedly a deficiency of the pituitary gland, administering *IGF 1* (Merimee et al. 1987), and a defect in the growth hormone receptor *GHR* (Merimee et al. 1989). *IGF 1* and *IGF 2* play an important role in swine growth processes as well (Van Laere et al. 2003; Jeon et al. 1999; Owens et al. 1990) and one first applications of gene editing on livestock was the knock-out of *GHR* in pigs, resulting in a drastic reduction of size (Cyranoski 2015). Former studies using SNP arrays on the GMP (Gaerke et al. 2014) identified additional candidate genes for growth, such as *SOCS2*, *TXN*, *DDR2* and *GRB10*. To this day, size inheritance remains poorly understood.

Next generation sequencing

The advent of molecular biotechnologies in livestock sciences changed the way animal breeders elucidate the genetic background of phenotypic traits. Today, SNP arrays are widely used, both for active breeding, as implemented in genomic selection, as well as for investigatory purposes, as in genome wide association studies (GWAS). While high-throughput genotyping arrays were still gaining importance in the aforementioned fields, the publication of several livestock genomes, beginning with the chicken genome in 2004 (Hillier et al. 2004), followed by the horse genome (Assembly 2007, Wade et al. 2009), the cow genome (Elsik et al. 2009), and the pig genome (Groenen et al. 2012), enabled the use of techniques collectively called ‘next generation sequencing’ (NGS) or ‘massively parallel sequencing’ (not to be confused with ‘third generation sequencing’). Different to SNP arrays, which rely on already known polymorphic positions and are specifically designed based on a predefined discovery set of animal samples, NGS is based on the re-sequencing of a whole genome. First sequencing approaches like Maxam-Gilbert- or Sanger-Sequencing were expensive and slow (by current standards) and were restricted to short sequences of specific loci. Sanger’s dideoxynucleotide sequencing became what is known as ‘first-generation-sequencing’ (Liu et al. 2012). This technique was used to produce the first *de-novo* sequence of the human genome (Lander et al. 1999) which took about 13 years and USD $100M. (NHGRI 2016). Second-generation sequencing or ‘pyrosequencing’ was introduced after a new mechanism of measuring pyrophosphate synthesis was discovered (Nyrén and Lundin 1985), that could visualize DNA synthesis in real-time without using radio- or fluorescently-labeled dNTPs and electrophoresis (Heather and Chain 2016). Both, Sanger- and pyrosequencing require DNA polymerase to synthesize the complement strand to the

respective DNA fragment and are therefore classed as ‘sequencing-by-synthesis’ methods. The term “next generation sequencing” was first coined, when sequencers reached the capacity to process millions of reads in parallel, enabling whole-genome association studies and other input demanding approaches (Reis-Filho 2009). Since then, the cost of sequencing has consistently dropped (**Figure 1.3**).

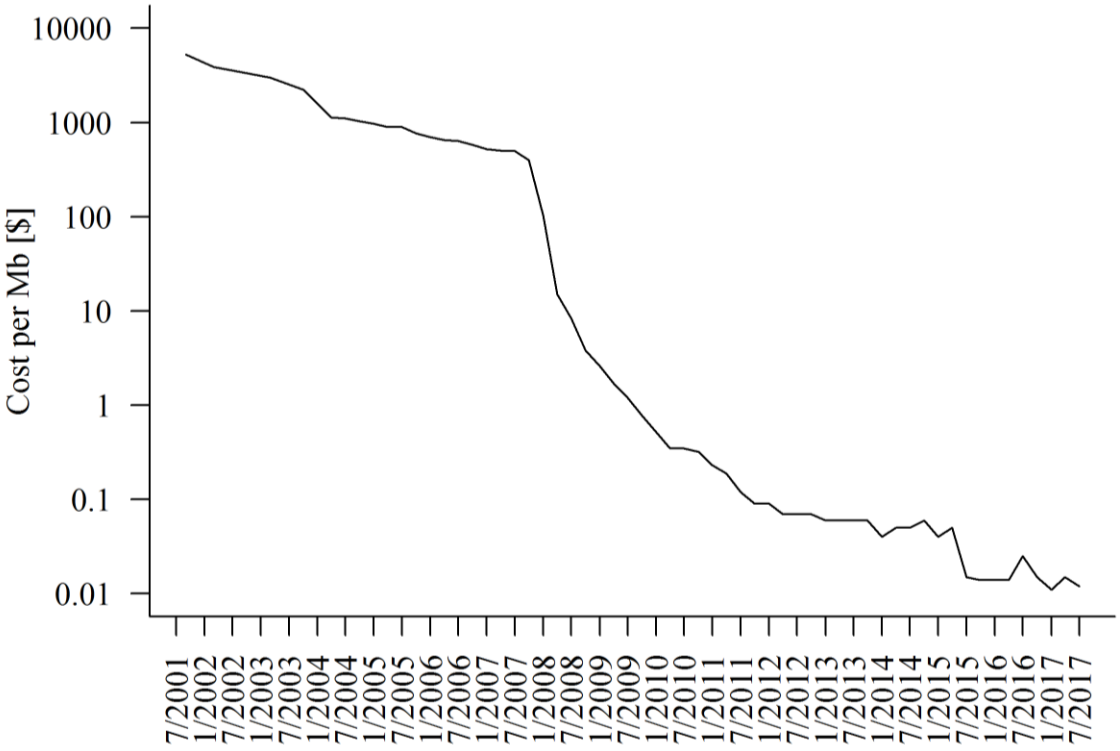


Figure 1.3: Sequencing costs per sequenced nucleotides (based on NHGRI 2016).

The first commercially successful massively parallel machine was developed by 454 Life Sciences and current NGS systems followed its concept, for example the Illumina HiSeq2000 and HiSeq X10, used in our studies.

The Illumina sequencing approach

Current Illumina sequencers use polymerase-based sequencing-by-synthesis with bridge amplification, that allows paired-end sequencing of short reads (Mardis 2008) and basic understanding of the principles of function is necessary to follow considerations made in this thesis. The following pipeline describes the workflow (Illumina 2018).

Library preparation

Extracted DNA is fragmented and tagged with adapters using transposons. Reduced cycle amplification is then used to add sequence primer binding adaptors, indices and a sequence

complementary to the oligonucleotides (oligos) on the sequencers flow cell, to each side of the read. Thus, the read can be tagged to the flow cell using the complementary regions, identified by the index, and sequencing can be initiated at the primer binding site.

Cluster Amplification

The bottom of each channel of the flow cell is covered by lawn of two types of oligos, which are complementary to the oligos, tagged to each side of the fragmented DNA. The fragments are washed over the channel and adhere to one type of oligos. The flow cell oligo sequence is then elongated by a polymerase along the original template. That double strand is denatured and the original template is removed. The template folds over to the second type of oligo, forming a bridge, during a process called bridge amplification. The complementary strand is synthesized along the bridge and the double-stranded bridge is denatured, resulting in single-stranded forward and reverse strand being hybridized to the oligos. This process is repeated over and over to produce millions of clusters of clones of each read. Eventually the reverse strands are removed from the flow cell, and the free complementary region of the forward strand is blocked to prevent undesired bridge amplification. At the end of clustering, the channel contains clusters of single forward strands fixed on the first type of oligos and free second type oligos.

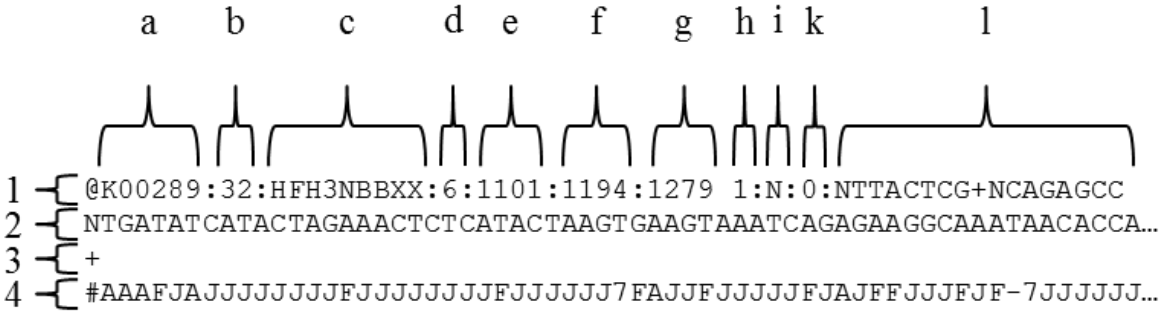
Sequencing

The sequencing primer is hybridized to the 5’ end. Fluorescently tagged nucleotides are provided and the complementary nucleotide is hybridized to the forward strand. The hybridized nucleotide emits a light signal which is detected and specific for each of the four possible nucleotides. Each Nucleotide is initially blocked to prevent hybridization of more than one base, ensuring, so that only one base is read per cycle. After a certain number of cycles (Our study: HiSeq2000: 100; HiSeqX10: 150) the double strand is denatured and the index primer is amplified. The index of the read is sequenced as the read before. Primer and index are denatured and the 5’ oligo region left unprotected, so the read can perform bridge amplification to the second type oligo on the flow cell. The second index is read to identify the two paired mate reads. The bridge is denatured and the forward read removed. The reverse read is sequenced as the forward read before.

Raw sequence preparation to variant calling

The output of the Illumina sequencers is provided in FASTQ format (Cock et al. 2010). The excerpt (**Scheme 1.1**) shows the four lines characterizing each read. For paired end sequencing, forward and reverse reads are normally separated into two files. The first line is a unique identifier, which enables traceability of every read ever produced by a sequencer. The second line is the nucleotide sequence of the read, the third line is always a '+' depicting connection of read and qualities, and in the fourth line are the respective Phred-qualities (Ewing et al. 1998) encoded as ASCII characters. Due to the sequencing technique, no further information about the origin of the read in the genome is provided, although necessary for further use in genomic studies. Therefor, reads are aligned to a reference genome of the respective species for downstream analysis.

Scheme 1.1: Excerpt from a fastq-file, produced by an Illumina HiSeqX10.



a	Instrument name	1	Identifier
b	Run ID	2	Nucleotide sequence
c	Flow cell ID	3	'+' connector
d	Flow cell lane	4	PHRED-scaled quality scores
e	Tile within flow cell		
f	X coordinate of cluster within tile		
g	Y coordinate of cluster within tile		
h	Pair mate number		
i	Read filter indicator		
k	Control bit		
l	Index sequences		

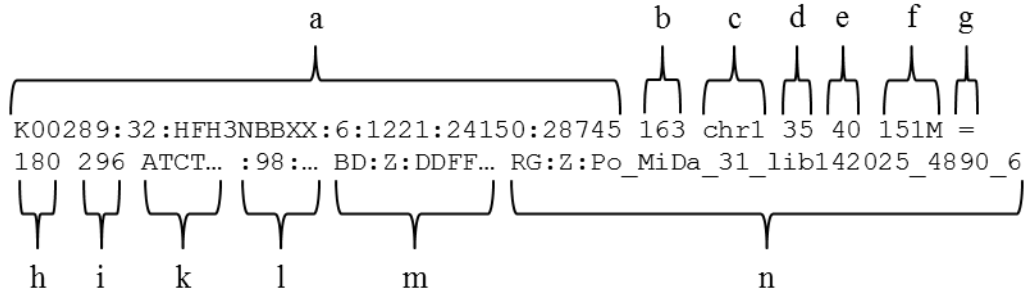
The official reference sequence Sscrofa10.2, lately superceded by 11.1, of the pig was assembled from BAC clone sequences and Illumina whole-genome shotgun reads of a female Duroc pig, named "TJ Tabasco" (Groenen et al. 2012). Alternatively, among others, a sequence assembly of a highly inbred Wuzhishan pig (Fang et al. 2012) was available at scaffold level (Access to all porcine assemblies: https://www.ncbi.nlm.nih.gov/).

Alignment

Alignment is the process of mapping every read to the reference genome. Since the appearance of next-generation sequence data, a range of programs have been developed for this purpose (Fonseca et al. 2012; Fonseca 2014). Among the most popular might be BLAST (Altschul et al. 1990), Bowtie (Langmead et al. 2009) and BWA (Li and Durbin 2009). The latter two are specifically designed to align NGS data. These programs have been constantly updated, and shown to be well balanced in terms of sensitivity, false positive rate, computation time and memory requirements (Otto et al. 2014; ECSEQ 2014). Both Bowtie and BWA rely on the Burrows-Wheeler pattern matching algorithm.

After alignment, read data is written to a file in sequence-alignment/map format (SAM, or BAM, which is the respective binary format; Li et al., 2009). The SAM-file (**Scheme 1.2**) contains mapping information about every read and is the basis for further analyses.

Scheme 1.2: Sequence-Alignment-Map format excerpt.



a	Read	h	Position of mate
b	Flag	i	Template length
c	Chromosome	k	Sequence
d	Position	l	Mapping qualities
e	Quality	m	Restored base qualities
f	CIGAR	n	Read group identifier
g	Chromosome of mate		

The SAM file contains information on the mapping position, mapping quality and the mapping of the respective mate pair. Each read can be traced back to the sequencing machine by the identifier and the unique read group ID assigned by the analyst, providing traceability downstream of the pipeline.

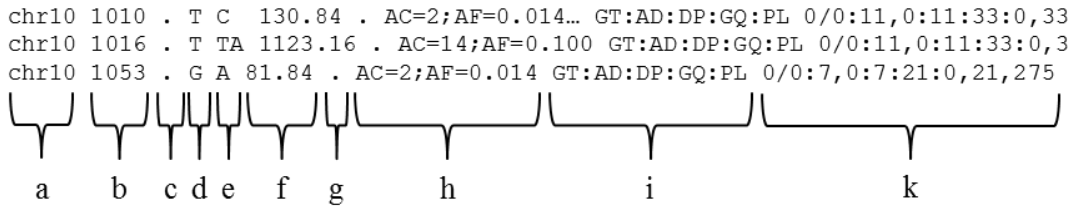
Aligned reads are initially unordered and further steps were required before variant calling, for example sorting of reads, merging, if a sample was sequenced in multiple libraries, marking of duplicated reads, and base quality recalibration. Prominent tools for data

preparation are for example Samtools (Li et al. 2009), Picard (Picard 2009), or the Genome analysis toolkit, GATK (Van der Auwera et al. 2013).

Variant calling

Variant calling is the process of identification of polymorphisms in the aligned sequence reads compared to the reference sequence. In the simplest case, the absence of sequencing errors and high read coverage, a variant would be every deviation from the reference sequence, found in the sequence reads, or for heterozygous loci, reads supporting any of two different alleles. A variant and genotype could easily be determined by counting alleles. Normally, both are done in two steps, where first a variable position is identified and then individuals' genotypes at the respective position are determined (Nielsen et al. 2011). Current variant callers, such as Samtools mpileup (Li et al. 2009), FreeBayes (Garrison and Marth 2012), or GATK haplotype caller rely on Bayesian methods, rather than simple allele counting. The output of variant callers is standardized in the Variant call format VCF (S**cheme 1.3**; Danecek et al. 2011)

Scheme 1.3: Example of three variants in the Variant-Call-Format (VCF).



a	Chromosome	f	quality
b	Position	g	Filter
c	SNP ID	h	Info field
d	Reference allele	i	Format
e	Alternative allele	k	Individual record

Variants can be filtered upon the attributes provided in the information field (h), to gain a reliable set for analysis. Filtering can either be based on independent thresholds for multiple attributes, such as mapping quality, strand bias or minimum call rate, or a machine learning algorithm can be trained on positions known as truly variable (Broad Institute 2017).

Signatures of selection

Interest in the genetic background of a trait, for example body size in pigs, leads to the question, if selection for the respective trait has shaped the underlying genomic region and

how this can be detected? The arrival of the NGS techniques described above, facilitated gathering information on the (almost) whole genome of an individual, enabling assessment of total genetic variation.

The theory of neo-darwinian selection states that a large proportion of variation has consequences for the fitness of the organism and most variants are therefore subjected to selective pressures (Nei 2005). In contrast, the neutral theory of selection (Kimura 1969) states that most of the variation in the genome is neutral and changes in allele frequency, or fixation, are in the most part due to genetic drift rather than selection.

In both theories, variable loci with effects on fitness, captured as the probability of an allele to be conveyed into the next generation, are prone to selective pressures, resulting in frequency changes of the favourable allele. In animal breeding scenarios, the chance to reproduce is highly dependent on an individual carrying a desired phenotype, and therefore being chosen for mating. The selection coefficient (Gillespie 2004), describes difference in fitness for two alleles by estimating the relative selective pressure against an undesired allele/genotype. While the selection coefficient can be relatively minor, as in the case of lactose tolerance (Bersaglieri et al. 2004), long term evolutionary pressures will eventually lead to fixation of the causative variant at the beneficial allele. This holds also if in the biallelic case the homozygous genotype is preferable. Dependent on whether the mutant allele or the ancestral allele are desired, selection is positive or negative, respectively, but both categories are counted as directional selection. In the case of over-dominance, the heterozygous genotype is favoured, and both alleles are maintained at intermediate frequencies, which is then called balancing selection. (Nielsen 2005).

Under directional selection, not only will the variant itself be fixed, but also the variant alleles in the vicinity of the selected allele will be co-selected due to genetic linkage. This is the so-called ‘hitch-hiking effect’ of the favourable gene (Smith and Haigh 1974). In the case of directional selection, this results in diminished variability around the selected locus. Such a region is called a ‘selective sweep’ (Pritchard et al. 2010) and is one case of a signature of past selection.

The selective sweep facilitates the identification of the location of the causative variant since neighboring variants exhibit similar genetic features as the causative variant. These features can be classified in the following categories:

Decrease in variability

Selection pressure will fix the favorable allele in the directional case, resulting in decreased nucleotide diversity (Nei and Li 1979) or decreased expected heterozygosity within the selective sweep (Smith and Haigh 1974).

Differentiation

When selection favours different alleles in different populations, allele frequencies at such a locus will diverge. The classic measure of differentiation between subpopulations is Wright's F_{ST}, introduced by Wright (1950). It is based on the inbreeding coefficient, which is defined as the probability of both alleles carried by an individual being identical by descent (Falconer and Mackay 1996) and its effect of shrinking heterozygosity. In the absence of inbreeding, the number of heterozygotes would be expected to be $2p(1-p)$ with p being the allele frequency of one allele (Weir 1996), but with inbreeding it is $2p(1-p) - 2Fp(1-p)$ with F being the inbreeding coefficient (Wright 1950). Another way to interpret F is as being the correlation between the two gametes of an individual (Holsinger and Weir 2009). Faced with the problem of inbreeding in sub-populations, Wright (1950) split the inbreeding coefficient F into three components F_{IT} (1), F_{ST} (2) and F_{IS} (3), which can be interpreted as co- ancestries (Holsinger and Weir 2009): (1) co-ancestry of the alleles of an individual in relation to the entire population ('inbreeding'), (2), co-ancestry of two randomly chosen alleles in a subpopulation in comparison to the entire population and (3), the co-ancestry of an individual's alleles relative to its sub-population. Even more simple, Hudson et al. (1992) define F_{ST} as $1 - \frac{H_w}{H_b}$, with H_w being the average number of differences between two sequences randomly sampled from the same sub-population and H_b being the average number of differences between sequences sampled from two sub-populations. The three F-values are interrelated as $F_{ST} = \frac{F_{IT} - F_{IS}}{1 - F_{IS}}$ (Wright 1950) which is equal to $(1 - F_{IT}) = (1 - F_{ST})(1 - F_{IS})$ (Weir and Cockerham 1984). F_{ST} is therefore a measure of differentiation between sub-populations. As a result, F_{ST} can also be used to detect diversifying or balancing selection between two subpopulations (Bowcock et al. 1991).

Since sequencing provides genome wide sets of variants, the F_{ST} distribution under neutrality no longer needs to be assumed or modeled, but can simply be quantified, and outliers in extreme tails of this distribution can be considered as candidate loci under selection (Akey et al. 2002). Also, neighboring F_{ST} values in regions under selection appear to be highly correlated and F_{ST} in coding SNPs has been found to be lower than at non-coding loci, which

may explain a functional constraint of these variant classes (Akey et al. 2002). F_{ST} is widely used in studies of selection in livestock (Leno-Colorado et al. 2017; Rubin et al. 2012; Wilkinson et al. 2013).

Linkage disequilibrium decay and number of haplotypes

In a finite population, favourable mutations are contained in a limited number of haplotypes. When selective pressure promotes a favourable allele, the respective haplotype or haplotypes are co-selected due to linkage which results in an increased level of linkage disequilibrium (LD; Barton 2000) that can be used to identify a selective sweep (Pérez O'Brien et al. 2014; Gholami et al. 2015). Prominent tests are EHH (extended haplotype homozygosity, Sabeti et al. 2002), which aims to identify highly frequent haplotypes that are longer than expected under normal recombination, or iHS (integrated haplotype score; Voight et al. 2006), which identifies loci where the derived allele is preferred and the respective haplotype is unexpectedly long. The latter is considered optimal to identify ongoing positive selection. Both measures have been extensively used to identify selective sweeps (Qanbari et al. 2011; Bomba et al. 2015).

Allele frequency spectrum

Under the neutral theory (Kimura 1991), it is expected that the number of polymorphisms at a site are in a relation to the number of pairwise differences between individual sequences at that site (Tajima 1989). This means, that if in a region with many segregating loci individuals differ at a relatively low number of these loci, this might be interpreted as a preference for certain haplotypes and therefore a sign of selection. The derived test, Tajima's D, provides a statistic that compares the mean number of differences to the number of segregating sites. It is scaled, so under neutrality the D-value is expected to be zero. A value below zero indicates less differences than expected, i.e. rare alleles at high numbers, which can be interpreted as a sign of positive selection, whereas a value higher than zero indicates unexpectedly high number of differences, i.e. an excess of common variants, being a sign of balancing selection.

Selection also shapes the allele frequency spectrum in a characteristic way (**Figure 1.4**) and modern tests aim to identify differences in the allele frequency distribution (Bustamante et al. 2001).

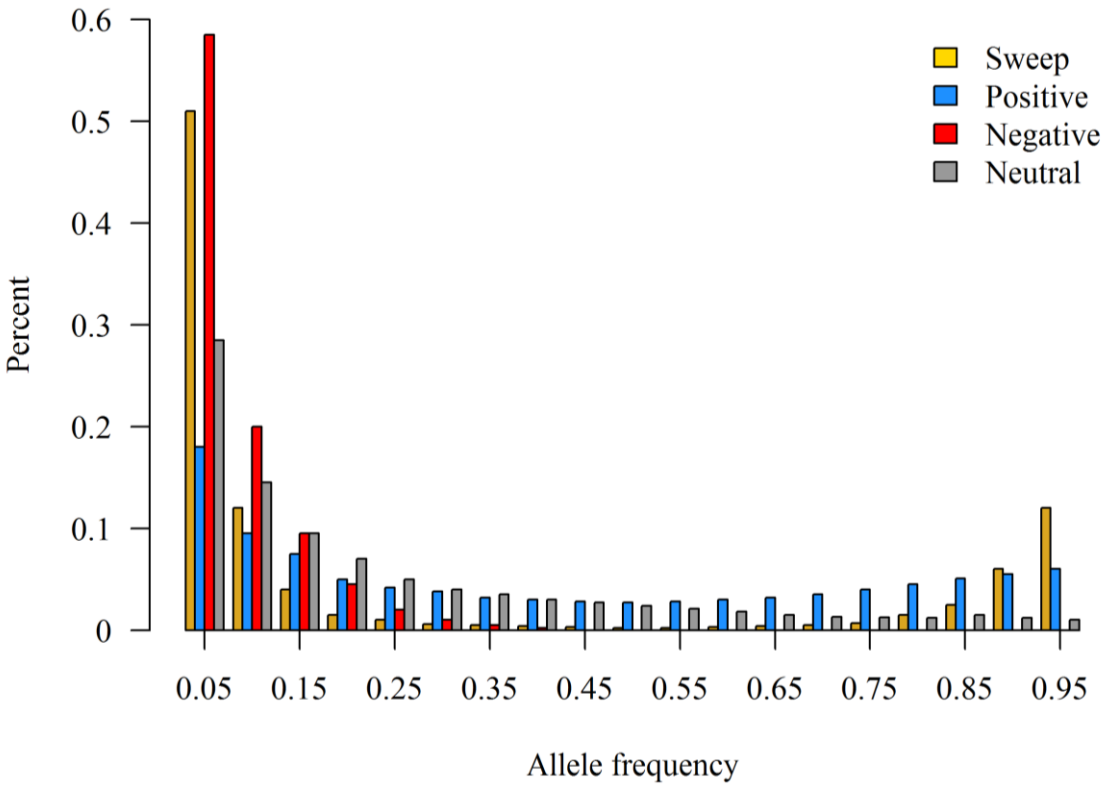


Figure 1.4: Allele frequency spectra in genomic regions under different types of selection (modified from Nielsen 2005).

A method based on composite likelihood (Kim and Stephan 2002), which compares the maximum composite likelihood estimated under a model of no selection against the composite likelihood under a model allowing selection, became prominent when detecting sweeps in the upcoming DNA data (Nielsen et al. 2005). This model was improved by replacing the composite likelihood of a model without selective sweeps by the composite likelihood estimated from the dataset itself (Nielsen et al. 2005). This approach also accounts for the ubiquitous problems of uncertainty in assumptions, such as recombination rates or population history when modeling and also ascertainment bias in the SNP data

McDonald- Kreitman-tests

The McDonald-Kreitman test evaluates the abundance of mutations in coding regions of genes (McDonald and Kreitman 1991). In principle, mutations in coding regions can be categorized into protein-changing non-synonymous mutations, and neutral synonymous mutations (Nielsen 2005). The assumption is that the ratio of substitutions of these two mutation types between species and the ratio of polymorphic mutations of both types within species should be balanced. Selection can alter those ratios since it is expected to affect the non-synonymous rather than the synonymous mutations. Depending on the type of selection,

positive or negative, the relative number of non-synonymous substitutions will either increase or decrease. This test can be enhanced by applying it to multiple sub-populations and comparing the ratios within a subpopulation and between the contrasts, to find genes under selection.

The statistics presented here, were chosen to give a notion of the basic principles of the detection of signatures of selection. Most scenarios hold for a hard sweep, a hitchhiking signature following to beneficial allele being swept through the population by selective pressure. But it should be mentioned, that there are also soft sweeps, not necessarily accompanied by a hitchhiking signature or polygenic adaptation (Pritchard et al. 2010). Additionally, numerous sophisticated statistics have been developed, sometimes by extending aforementioned methods by cross-population testing, e.g. XP-EHH or XP-CLR (Sabeti et al. 2007; Chen et al. 2010), sometimes combining known approaches (Grossman et al. 2010; Ma et al. 2015).

Functional annotation

Approaches, like the aforementioned McDonald-Kreitman test require precise knowledge if a mutant allele is synonymous or non-synonymous. On the other hand NGS studies produce vast amounts of data, with millions of variants being discovered, normally in the form of SNPs or short insertions or deletions (InDels). Obviously, an individual evaluation of each variant is impossible and more efficient approaches are needed (Wang et al. 2010a). Approximately only 1.2 % of mammalian genomes represent coding regions (Human Genome Sequencing Consortium 2004). Common annotation tools, e.g. ANNOVAR (Wang et al. 2010a), use gene databases such as Ensembl (Aken et al. 2016) to determine if a variant is located in a coding region or, for example, in between of genes. They also incorporate annotated mRNA sequences and known variants, and can be used for analyses involving livestock, although this information is mostly derived for model organisms, such as humans or mice. It is well understood which amino-acid a codon-triplet of respective mRNA is translated in protein-biosynthesis (Matthaei and Nirenberg 1961; Nirenberg and Matthaei 1961), but this does not predict if the replacement of an certain amino acid has functional constraint on the resulting protein. Therefore, approaches such as SIFT (Sorting-Intolerant-From-Tolerant; Ng and Henikoff 2003) and GERP (Genomic Evolutionary Rate Profiling; Cooper et al. 2005) have been developed. Both assume that an amino acid change is more likely to have functional consequence when it is highly conserved in homologous sequences, derived from related protein sequences (SIFT; Ng and Henikoff 2001) or multiple sequence

alignment of a set of related species from the same class, e.g. mammals (GERP; Cooper et al. 2005). Besides relatively easily identifiable coding mutations, as described before, it is known that there are various other mutations with functional consequence. Initiatives such as FAANG aim at further characterizing these variations in livestock (Andersson et al. 2015).

Objective and aim

Marker based approaches, microsatellites and SNP-arrays, have been utilized in research in livestock for many years (Womack 2005). The arrival of affordable massively parallel sequencing offers new opportunities to reveal the genetic basis of interesting traits. Thus, in theory causal variants can be identified directly, rather than just via their hitchhiking effect on surrounding markers, especially employing recent developments such as reverse genetics. Recent studies have proven that analysis of NGS data is a powerful means to elucidate the genetic background of phenotypically complex traits. Prominent examples are gait patterns in the horse (Andersson et al. 2012), comb morphology in the chicken (Imsland et al. 2012) and coat colour in the swine (Rubin et al. 2012). Another feature of NGS variant sets is that they are relatively less affected by ascertainment bias than SNP-arrays, which are suited for a specific set of discovery populations (Malomane et al. 2018), and could therefore be used to calculate unbiased estimates of variation and differentiation in breeds not in the discovery set.

The Goettingen Minipig as a highly controlled breed of exceptionally small body size, is a highly promising candidate to eluciade the genetics behind miniaturization in pigs. For several reasons, it has been bred in separated stocks. While the breeding programme focuses on the management of inbreeding and minimisation of population divergence, the processes that have influenced the genome as a result of the separation of the breeding units are of particular interest to the breeders.

This study aims to use whole-genome re-sequencing to characterize the following fundamental aspects relating to the Goettingen Minipig genome:

1. What is the genetic background of the body size difference between conventional fattening pigs and two breeds of minipigs, the GMP and the MiniLEWE?

2. Is there stratification between isolated GMP breeding stocks, and could the high resolution and low ascertainment bias of NGS data enhance its assessment?

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CHAPTER 2

The Minipig Genome Harbors Regions of Selection for Growth

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Abstract

The whole genome resequencing (WGS) data of 46 normal sized pigs, either domestic or wild, was compared to WGS from 11 Göttingen Minipigs, 2 Berlin Minipigs, 2 Xiang pigs and one DNA pool comprising 10 Berlin Minipigs. Expected heterozygosity in the minipigs and fixation between both groups where used as a measure to find selective sweeps introduced during the selection for low body size in the minipig. 166 such candidate regions were defined and further annotated. Gene Ontology overrepresentation analysis revealed significant enrichment of terms related to growth. A large set of contained genes has been found, which have influence on i.e. growth and bone development. *TGFβ* and plenty of its altering genes were identified.

Keywords: minipig, sequencing, growth

Introduction

The Göttingen Minipig (GMP) is one of the smallest pig breeds in the world. It was bred at the University of Göttingen, Germany, in the 1960´s to fulfill the rising needs for laboratory animals (Simianer and Köhn 2010). The Vietnamese Potbellied Pig, the Minnesota Minipig and the German Landrace were used as founder breeds. Intense selection led to a white-coated animal with less than 45 kg at an age of two years. This constitution makes it a promising candidate to reveal the genetic basis of growth and body size when compared to normal sized pig breeds.

A previous study (Gaerke et al. 2014) using 60 k SNP data revealed that alleles from all founder breeds can still be found in the genome of the GMP, but the proportions deviated significantly from the composition expected from the pedigree. Extreme differences between expected and observed breed composition in some genomic regions can be attributed to selection for low body weight and white skin color. These signatures of selection occur in regions where genes with known relevance for growth (e.g. SOCS2, TXN, DDR2 and GRB10) are located. Another finding was that information derived from the 60 k SNP markers is not sufficient to make a reliable statement on the genetic background of small body size in miniature pigs.

Next Generation Sequencing (NGS) technology provides the possibility to obtain whole genome data from many individuals at a reasonable price. The porcine reference genome was published in 2012 (Groenen et al. 2012) and first studies (Rubin et al. 2012) suggested that whole genome resequencing is a viable approach to identify regions under anthropogenic selection, since this method provides a much more comprehensive insight into genomic

variability based on SNPs and other types of variation such as structural variants than do SNP arrays. Even causal mutations have been derived from this data directly (Andersson et al. 2012; Imsland et al. 2012). However, minipigs have not been included in any of these studies so far.

Materials and Methods

Public Data. From the European Nucleotide Archive (ENA) sequence data from 37 domestic pigs, 11 wild boars from Asia and Europe, respectively, underlying the study of Rubin et al. (2012) and a Göttingen Minipig (Vamathevan et al. 2013) were downloaded.

Minipig Sampling. Blood samples were obtained from 10 individuals from the University owned stock and 2 individuals from the Berlin Minipig housed at the University of Veterinary Medicine, Hannover. A DNA pool from 10 Berlin Minipigs was added. All samples were sequenced with 10X coverage on the NGS-Platform at Uppsala University.

Basic Data Preparation. Raw sequence data was aligned to the Sus Scrofa 10.2 reference genome (Groenen et al. 2012) using BWA (Li and Durbin 2009), were sorted by Samtools (Li et al. 2009) and duplicates were marked with Picard tools (Picard 2009). Finally SNPs were called using the GATK (DePristo et al. 2011; McKenna et al. 2010).

Filtering. First indels and non biallelic SNV were discarded. In the second step SNP sets were filtered to remove unreliable SNP calls. Therefore SNPs in clusters with >5 SNPs in 20 basepairs, with BaseQualityRankSum <-5.5 or >5.5, MappingQualityRankSum <-11 or >11, ReadPosRankSum <-6, FisherStrand values >45, a Mapping Quality <30, and a Depth of Coverage <90X or >840X were discarded. To pass subsequent genotype filtering an individual needed a genotyping quality >20 and a pool needed a coverage >4 reads at this position.

In silico pooling. To avoid an over-influence of highly represented breeds, animals of these breeds were pooled. For each locus, the mean reference allele frequency was calculated, and only loci with at least a 50% genotyping rate were included. Afterwards, two contrasting groups (minipig vs. normal sized pigs) were formed. The *in-silico* pooled minipig group contained the information of 11 Göttingen Minipigs, 2 Berlin Minipigs, the Berlin Minipig pool and two Xiang pigs from China, which turned out to actually be minipigs (Zhang et al. 2005)

Genome wide scans. To determine regions where minipigs are differentiated from the normal sized pigs, F_{ST} values (Weir 1996)were calculated between the two groups. In order to find regions in the minipig genome where selective pressure for low body size massively shrunk the variability of many loci, expected heterozygosity H_{exp} in the minipig pool was calculated and normalized via a z-transformation. Both measures were subsequently summarized in 20 kb windows with an overlap of 50 %. Stringent criteria were used to define clear borders of regions with a certain pattern of an excessive overrepresentation of high F_{ST} values or low H_{exp}, in order not to rely on a simple extreme value approach. Every region with low H_{exp} which overlapped with a region of high F_{ST} was considered to be a selective sweep and intersected with the Ensembl Biomart Pig Gene set (Flicek et al. 2014). Gene enrichment analysis with Fisher's exact test and a $\chi^2 -$ test was performed on all GO terms found in the defined regions. The aberrant site frequency spectrum method (Nielsen et al. 2005), implemented in Sweepfinder was performed to add support to our custom approach.

Results and Discussion

Variant and sweep discovery. After variant calling and filtering, 35 million SNPs on the 18 autosomes and the X-chromosome formed the basis for later analyses. In the minipig a total of 20 million SNPs were found. Combining reduced heterozygosity and high differentiation between minipigs and normal-sized pigs revealed 166 candidate selection regions, summing up to 15.7 % of the pig genome. **Figure 2.1** shows two sweep regions on chromosome 9 and 6, respectively. It can be clearly observed, that both a relatively high F_{ST} and a low heterozygosity value are needed to define a sweep Nearly every sweep detected by Sweepfinder could be confirmed by this method, but in turn only a part of our candidate regions were detected by Sweepfinder. Such an example is the presented sweep on chromosome 9, where Sweepfinder produces just a weak signal, but a clear pattern can be observed from the other measures. Gene overrepresentation analysis for these regions gave 181 significant GO-Terms at a p-value <5 %. The best hits regarding the search term 'growth' are listed in **Table 2.1**. It should be mentioned, that the first hit was 'hormone activity' followed by 'response to glucose stimulus'.Genes connected to these pathways and found in a sweep region were for example *TGFβ*, which seems to play a key role for growth, as described by Enayati and Rahimi-mianji (2009) who detected an influence on the growth of hens. *SMAD7* (Nakao et al. 1997), *LEMD3* (Lin et al. 2005), *BAMBI* (Sekiya et al. 2004), *SKIL* (Tecalco-Cruz et al. 2012), and *MSTN* (Hickford et al. 2010) are known to assist *TGF*.

Stratil et al. (2006) found a growth QTL in the *ASPN* gene and (Labrador et al. 2001) found, that an elimination in the *DDR2* gene leads to dwarfism in mice.

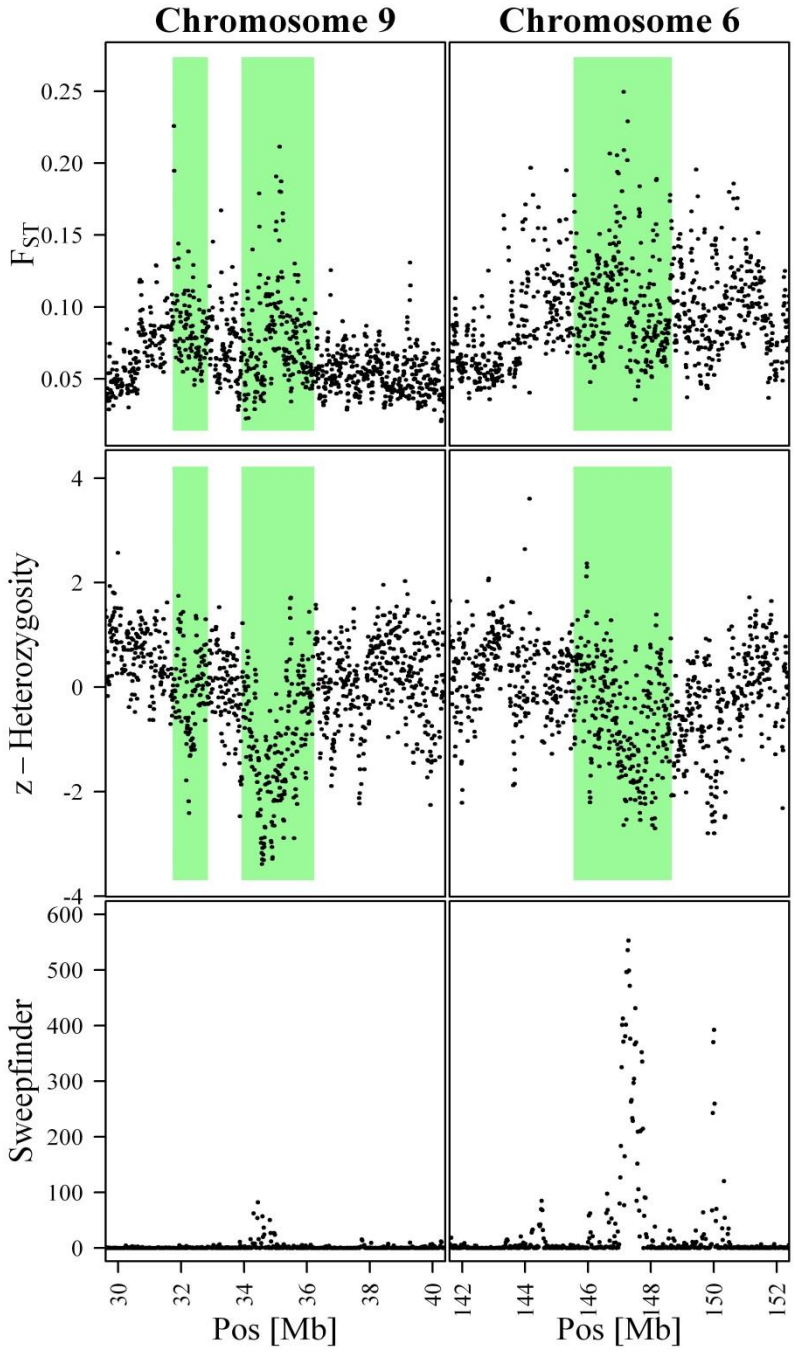

Figure 2.1: Fixation index FST and z-transformed heterozygosity values with underlying identified sweep regions and Sweepfinder composite likelihood ratio in 20 kb windows, overlapping by 50% in two regions on chromosome 9 and 6.

Table 2.1: Growth linked GO terms.

GO	P	GO Description
0071363	0.018	cellular response to growth factor stimulus
0001832	0.027	blastocyst growth
0035264	0.053	multicellular organism growth
0003416	0.072	endochondral bone growth
0036120	0.072	cellular response to platelet-derived growth factor stimulus
0045927	0.072	positive regulation of growth
0008083	0.079	growth factor activity
0030512	0.087	negative regulation of transforming growth factor beta receptor signaling pathway

GO: Biomart GO-Term accession, P: Fisher´s exact test p-value

Conclusion

By using variation data from whole genome resequencing even narrow sweep regions can be detected, just by the right combination of simple measures. The contrast of several normal sized and several minipig breeds increased the chance of finding differentiation associated with growth and size only. Annotation with Ensembl Genes and enrichment analysis revealed a sensible set of genes related to growth. *TGFβ* and Genes which are known to have influence on it seem to play an important role in the search for the genetic basis of low body size in pigs.

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CHAPTER 3

Analysis of porcine body size variation using re-sequencing data of miniature and large pigs

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Abstract

Background: Domestication has led to substantial phenotypic and genetic variation in domestic animals. In pigs, the size of so called minipigs differs by one order of magnitude compared to breeds of normally sized pigs. We used biallelic SNPs identified from re-sequencing data to compare various publicly available wild and domestic populations against two minipig breeds to gain better understanding of the genetic background of the extensive body size variation. We combined two complementary measures, expected heterozygosity and the composite likelihood ratio test implemented in "SweepFinder", to identify signatures of selection in Minipigs. We intersected these sweep regions with a measure of differentiation, namely F_{ST}, to remove regions of low variation across pigs. An extraordinary large sweep between 52 and 61 Mb on chromosome X was separately analyzed based on SNP-array data of F_2 individuals from a cross of Goettingen Minipigs and large pigs.

Results: Selective sweep analysis identified putative sweep regions for growth and subsequent gene annotation provided a comprehensive set of putative candidate genes, including *MAPK1* and *PPARG*. A long swept haplotype on chromosome X, descending from the Goettingen Minipig founders was associated with a reduction of adult body length by 3 % in F_2 cross-breds.

Conclusion: The resulting set of genes in putative sweep regions implies that the genetic background of body size variation in pigs is polygenic rather than mono- or oligogenic. Identified genes suggest involvement of the MAPK pathway and a possible insulin resistance to play a key role in miniaturization. A size QTL located within the sweep on chromosome X is, with an estimated effect of 3 % on body length, comparable to the largest known in pigs or other species. The androgen receptor *AR*, previously known to influence pig performance and carcass traits, is the most obvious potential candidate gene within this region.

Keywords: Goettingen Minipig, whole genome resequencing, body size, X-chromosomal QTL

Background

The livestock species of today display vast phenotypic variation. Domestication has shaped these species by increasing the variation in traits related to, performance, fitness, morphology and appearance, thereby changing the - phenotypically rather uniform - wild ancestors to the illustrious collection of our modern breeds. Focusing on body size, Haldane (1927) discussed a general principle why the horse is larger than the rabbit, or the cow is larger than the pig,

and suggested that there must be a right size for a certain form of a body and a change in size must be accompanied with a change in form. In contradiction to that, we see a wide range of body size or weight in just one species. Taking the example of pigs (*Sus scrofa*), the process of domestication of the wild boar led to animals that span from large fattening pigs to the so called 'miniature pigs' or simplified 'minipigs'. Their sizes differ by up to one order of magnitude. Among the minipigs the Goettingen Minipig (GMP) is one of the smallest breeds under a stringent breeding scheme (Simianer and Köhn 2010; Swindle et al. 2012). The Goettingen Minipig is a composite breed developed in the 1960's at the former Institute of Animal Breeding and Genetics at the Georg-August-University Göttingen in Germany. It was founded by crossing Minnesota Minipigs (MMP) with Vietnamese Potbellied Pigs (VPP). Later German Landrace pigs (LAR) were introduced to produce uniformly white animals (Glodek and Oldigs 1981). This pig breed shows a form of miniaturization called "proportional dwarfism" and Simianer and Köhn (2010) suggested that this is a form of pituitary dwarfism, caused by lower secretion of growth hormones from the pituitary gland, leading to a decreased secretion of the insulin-like growth factor 1 (*IGF1*).

The availability of porcine SNP chips offers the possibility to screen the genome for regions carrying genetic variants associated with the reduced size of minipigs. Gaerke et al. (2014) conducted a study on signatures of selection in GMP, MMP, VPP and LAR, using a 60k SNP chip. They found that alleles from all founder breeds were still segregating in the GMP and identified numerous putatively positively selected regions in the GMP. They suggested that a pathway connecting *SOCS2* and *GRB10* with *IGF1* could exist that plays an important role in the dwarfism of the GMP. Due to the limited marker density of the SNP array it was not possible to reveal causal mutations.

The current reference genome is based on the sequence of a Duroc pig and the first studies, using this reference to provide insight into the porcine demography and evolution (Groenen et al. 2012) and into the patterns that domestication and anthropogenic selection have left in the porcine genome (Rubin et al. 2012), were published in 2012. While these studies used diverse sets of pig breeds from all over the world, minipigs were not included. The very same month, the genome of a highly inbred Chinese Wuzhishan minipig was published (Fang et al. 2012) as an additional reference genome for Asian pigs, which have been domesticated independently from the European pigs (Giuffra et al. 2000). The present study aims at comparing WGS data of a diverse set of pig breeds to unveil the genetic mechanisms behind body size variation, and more specifically the miniaturization in pigs. Towards this aim, we

compared a group of miniature pig breeds to a group of large pig breeds by screening for highly differentiated regions under selection in the minipigs. Such candidate regions were subsequently screened for candidate genes with a putative effect on growth or body size, and the postulated effects on body-size of one of the identified candidate region was confirmed with data of an independent crossbreeding experiment.

Results

Number of SNPs

Biallelic SNPs are the most common class of variants used in genetic studies of animal genomes. Due to the explorative nature of SNP calling from WGS data, the number of SNPs is an indicator of variability in the analysed dataset, but also of strictness of the variant discovery and filtering. SNP calling from the DNA sequencing data revealed 46×10^6 biallelic SNPs genome-wide, of which 29×10^6 were polymorphic or fixed for the alternative allele in the minipigs. After filtering, 35×10^6 loci remained for all samples and 19.8×10^6 for the minipigs, respectively, 19×10^6 in the European domestics, 9.4×10^6 in the European wild boars, 19.5×10^6 in the Asian domestics and 19.2×10^6 in the Asian wild boars. Subsequent in-silico pooling left 27.6×10^6 loci with sufficient information to compare minipigs against large pigs.

Phylogeny

When comparing large pig breeds to minipigs, it is important to account for stratification within each contrasting group to ensure, that no breed specific signals will be identified. Therefore each group should be made up from phylogenetically different breeds, which in an optimal case share just the small or large body size, respectively. The analysis of genetic distances between sampled breeds revealed a clear division of European and Asian large pigs, with minipigs clustering closer to the Asian pigs (**Figure 3.1**). Estimation of F_{ST} also showed that the minipigs were closer to the Asian breeds than to the European breeds (F_{ST} = 0.08 and 0.12, respectively), while both minipig breeds were marginally closer to the domestic groups of both continents than to the respective wild boars. This effect is smaller for the GMP (GMP to European domestic/ wild: 0.14, 0.16; GMP to Asian domestic/ wild: 0.10, 0.11), whereas there is clear distinction for the BMP, which is much closer to both domestic groups than to the wild boars (BMP to European domestic/ wild: 0.07, 0.14; BMP to Asian domestic/ wild: 0.08, 0.11). The F_{ST} value between both minipig groups is 0.09. The highest differentiation

overall has been estimated between European and Asian wild boars (additional information in **Supplementary table 3.1** and **Supplementary table 3.2**).

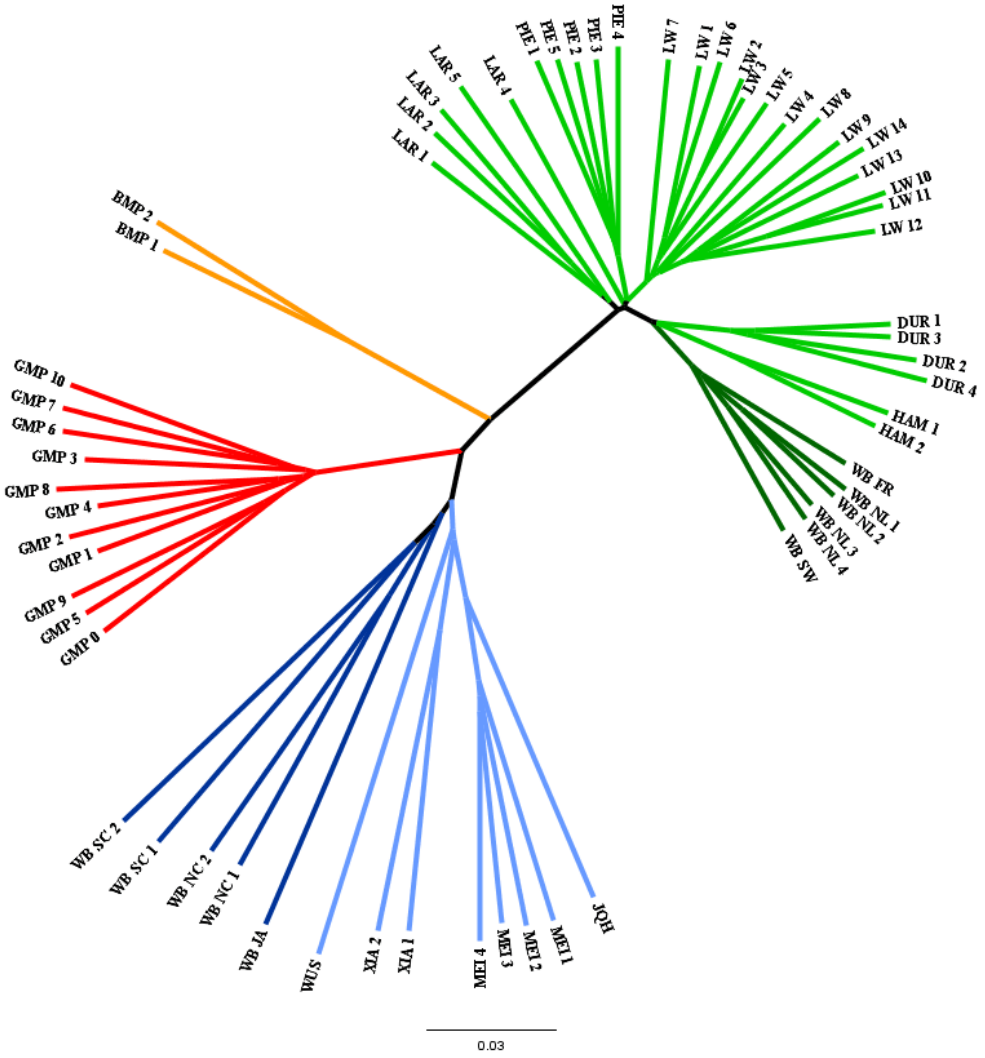

Figure 3.1: Neighbor-joining tree computed from pairwise IBS distances. Based on SNP data of the randomly selected chromosomes 1, 8 and 13 for all individuals (due to computational limitations). Asian wild boars in dark blue, Asian domestics in light blue, European wild boars in dark green, European domestics in light green, Mini-LEWE in orange and Goettingen Minipigs in red.

Selective sweeps

We searched for genomic regions under selective pressure for body size using a so-called selective sweep analysis and subsequently identified candidate genes within these regions. Further, the Gene Ontologies (GO´s), which represent functional categories, linked to every detected gene were checked for over-representation of certain GOs within sweeps compared

to the unselected background, to identify functional categories rather than single candidates. The selective sweep analysis revealed considerable parts of the genome as putatively being targeted by selection for growth. Not every chromosome was affected equally. Most of the 49 identified signals extended between 1 Mb and 2.5 Mb, but one on chromosome 14 reached nearly 10 Mb. The other large signals were located on chromosomes 5 (2.8 and 4.3 Mb), 8 (4.6 Mb), 13 (5.2 and 2.9 Mb), 14 (3.6 Mb) (**Figure 3.2**) and chromosome X (48 Mb; not shown). SweepFinder detected fewer, but larger regions, whereas the regions detected by decreased heterozygosity were more numerous but smaller. The exceptionally large region on chromosome 14 consists of an accumulation of many small signals reflecting reduced heterozygosity and two large signals from SweepFinder. The union of both signals gives a nearly uninterrupted huge selective sweep signal.

Genes in Sweeps and their functions

The Ensembl porcine gene set 79 annotation within sweep regions on the autosomes revealed 524 genes (**Supplementary table 3.3**).

Gene ontology over-representation

In total, we analyzed 2006 unique GO terms linked with genes located in putative sweep regions. 55 of these gene ontologies were found to be significantly overrepresented within sweeps by using a Fisher´s exact test P-value lower than the 5 % quantile threshold of the empirical distribution function for the respective ontology. **Table 3.1** shows a selection of gene ontologies over-represented in putative sweeps (see also **Supplementary table 3.4**).

A literature review for all genes belonging to statistically significant GO terms with a focus on properties characterizing minipigs revealed a comprehensive set of genes with interesting putative functions (**Table 3.2**). Among them are genes like *COMT* and *PATZ1*with direct effects on growth or size in other organisms, *ACOT4* and *PKP2*, which are involved in growth factor signaling, or genes directly linked to growth in swine, for example *PPARG* that is suspected to be a key factor in porcine growth, conformation and fatness. Additionally, we found a considerable number of genes with links to the MAPK signaling cascade, e.g. *MAPK1* and *PTPRR*, involved in glucose and lipid metabolism, or putatively responsible for insulin resistance or diabetes type II or obesity.

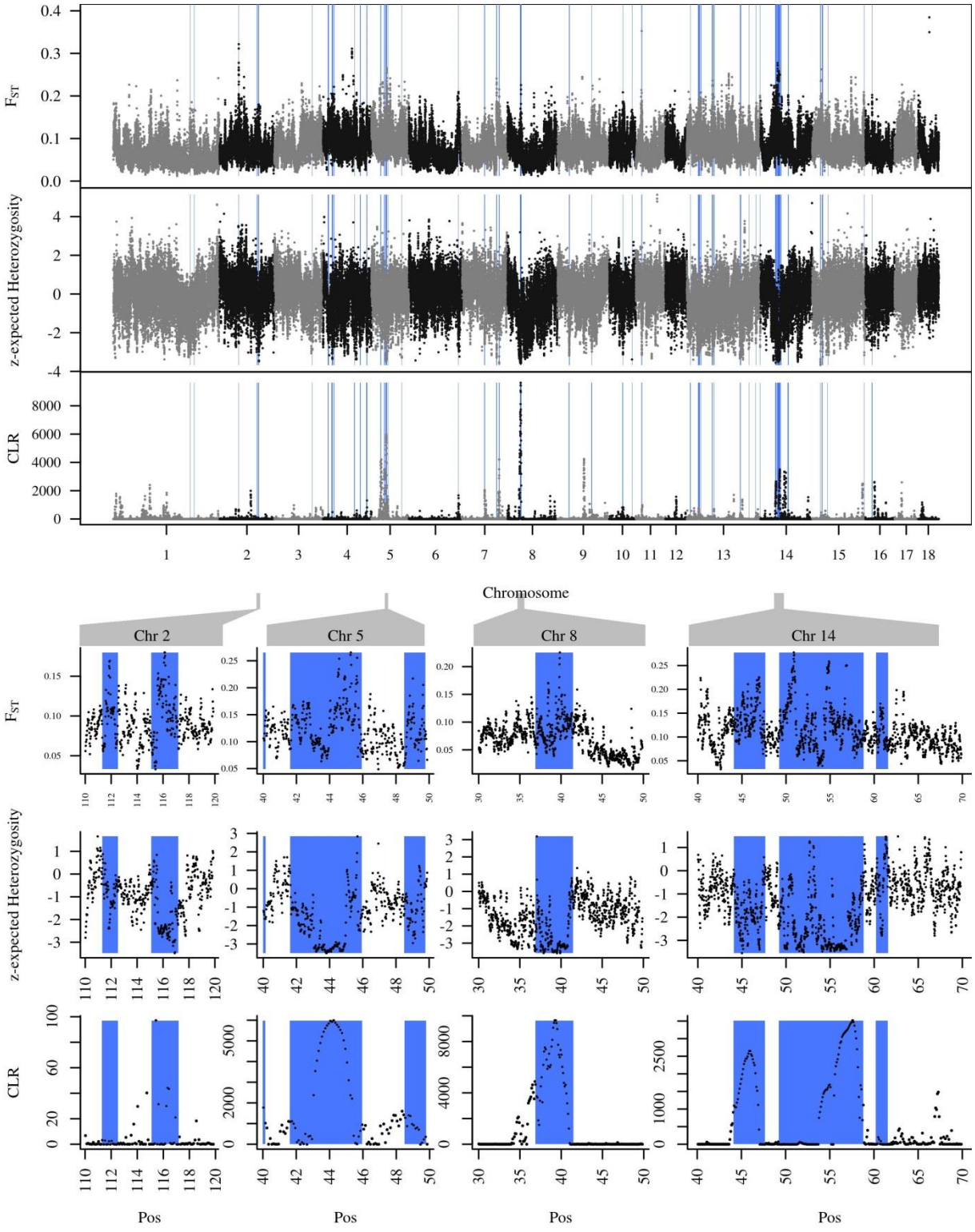

Figure 3.2: CLR test and normalized expected heterozygosity within minipigs and FST between large pigs and minipigs. Regions on chromosomes 2, 5, 8 and 14 identified as putative selective sweeps are highlighted; Blue rectangles underlie detected putative sweeps.

Table 3.1: Selected gene ontologies over-represented in putative sweeps.

No.	Fisher-P	Empirical p-value	Number of genes in term and sweep	Fold Enr.	GO Term Name
1	0.0017	0.0002	7	3.94	Z disc
2	0.0012	0.0014	4	0.26	negative regulation of transcription from RNA polymerase II promoter
3	0.0050	0.0040	5	4.38	protein tyrosine/serine/threonine phosphatase activity
4	0.0172	0.0059	4	3.94	Microvillus
5	0.0060	0.0063	4	5.25	regulation of alternative mRNA splicing, via spliceosome
6	0.0149	0.0067	13	1.99	mitochondrial inner membrane
7	0.0033	0.0067	10	2.75	protein dephosphorylation
8	0.0024	0.0078	54	1.52	Mitochondrion
9	0.0096	0.0081	3	6.44	leukocyte tethering or rolling
10	0.0125	0.012	3	5.91	ventricular cardiac muscle cell action potential
22	0.0272	0.0222	10	2.13	actin cytoskeleton
25	0.0101	0.0239	2	11.81	mitochondrial electron transport, ubiquinol to cytochrome c
27	0.0101	0.0248	2	11.81	positive regulation of growth
31	0.006	0.0286	4	5.25	social behavior

Table 3.2: Candidate genes from significant ontologies with putative functional link to minipigs.

Gene name	Function	Reference
ACACB	Downregulated by *TGFB1*; influencing type-II-diabetes; obesity and lipid metabolism	Zhou et al. 2005; Ma et al. 2013
ACOT4	Linked to *FGF21 in mice*	Muise et al. 2013
ADAMTS12	Blocks Ras/ MAPK pathway	Llamazares et al. 2007
COMT	Reduced birth weight in humans	Sata et al. 2006
DUSP28	Activator of MAPK pathway	Wang et al. 2014
HYAL1, *HYAL2*	Overexpressed in the placenta of the smallest pig fetuses	Vallet et al. 2010
LTBP1	*TGFB* signaling, role in the regulation of human height	Lango Allen et al. 2010
MAGOH	Influences MAPK	Roignant and Treisman 2010
MAPK1	Coding central proteins *ERK2* in the Ras/ MAPK	Reviewed by Cobb et al. 1991
NDUFB9	Severe growth-hormone deficiency	Riedl et al. 2004
OSM	Diabetes type II	Sanchez-Infantes et al. 2014
PATZ1	*PATZ1*-null mice were retarded in growth, Homozygote animals were 10 to 20 % smaller, than their litter mates of the same sex	Valentino et al. 2013
PKP2	Associated to *EGF*	Kazlauskas 2014
PPARG	Muscle specific expression; deletion causes insulin resistance in mice; key role in pig growth; reduced size in pre-pubertal children	Crooks et al. 2014; Hevener et al. 2003; Puig-Oliveras et al. 2014; Cecil et al. 2005
PRKAR2A	Obesity and lipid metabolism	Park et al. 2012
PTPRR	Member of the MAPK pathway	Hendriks et al. 2009
SOD1	Depressor of the MAPK pathway central genes *ERK1/2*	Juarez et al. 2008

Strong selective sweep on chromosome X

The major selective sweep on chr. X known from the studies of Rubin et al. (2012) and Ai et al. (2015) is also found in the minipigs. It is known that this sweep consists of two majorly un-recombining haplotypes of about 9 and 39 Mb, respectively. **Figure 3.3A** shows a substantial decrease of the expected heterozygosity within the minipigs in a 48 Mb region in the middle of chromosome X between 52 Mb and 100 Mb. The fixation index shows that this region consists of two separate sub-regions. The first part, approximately inside the interval 52 Mb to 61 Mb, appears to be unique to the minipigs, whereas the moderate level of differentiation in the second part implies that the minipigs are similar to some breeds of the large pig group. We postulated that this genomic region might have an effect on body size and therefore utilized data of a former cross-breeding experiment, to estimate QTL effects for each existent haplotype.

The phylogenetic tree of all sequenced animals based on all markers inside the first region (**Figure 3.4**) shows that the haplotype carried by the minipigs is shared with only the Xiang pigs and two wild boars from South China. The sub-tree for the second region clusters the samples into two main groups, the first comprising the minipigs, the Xiang, the Meishan, the Jiangquhai and the South Chinese wild boars, and the second all European breeds and the wild boars from North China and Japan.

Analysis of SNP chip data

Since the haplotype carried in the region chrX:52- 61 Mb appears to be typical for minipigs, we used genotyping data from two former studies (Gaerke et al. 2014; Pant et al. 2015) to determine the haplotypic state of animals with recorded phenotypes in order to enable the estimation of the effect of the minipig haplotype on size. The Illumina PorcineSNP60 BeadChip contains 23 SNPs located on chromosome X between 52 and 61 Mb according to the current map based on the genome build 10.2. Filtering removed 7 individuals for poor genotyping (call rate < 10 %), 3 SNPs that were missing and 13 SNPs, which had a low minor allele frequency. 8 SNPs (**Supplementary table 3.5**) passed the filtering, three of them in the beginning of the region around 53 Mb (MARC0056564, MARC0046345, H3GA0051807), three in the center around 57 Mb (INRA0056742, H3GA0051810, MARC0013223) and two at the end around 60 Mb (INRA0056744, H3GA0051814). At the first three loci, all minipigs carry a guanine, a cytosine and a guanine, respectively, but also two Duroc females from the Danish study are heterozygous and convey this allele to the subsequent generations of cross-

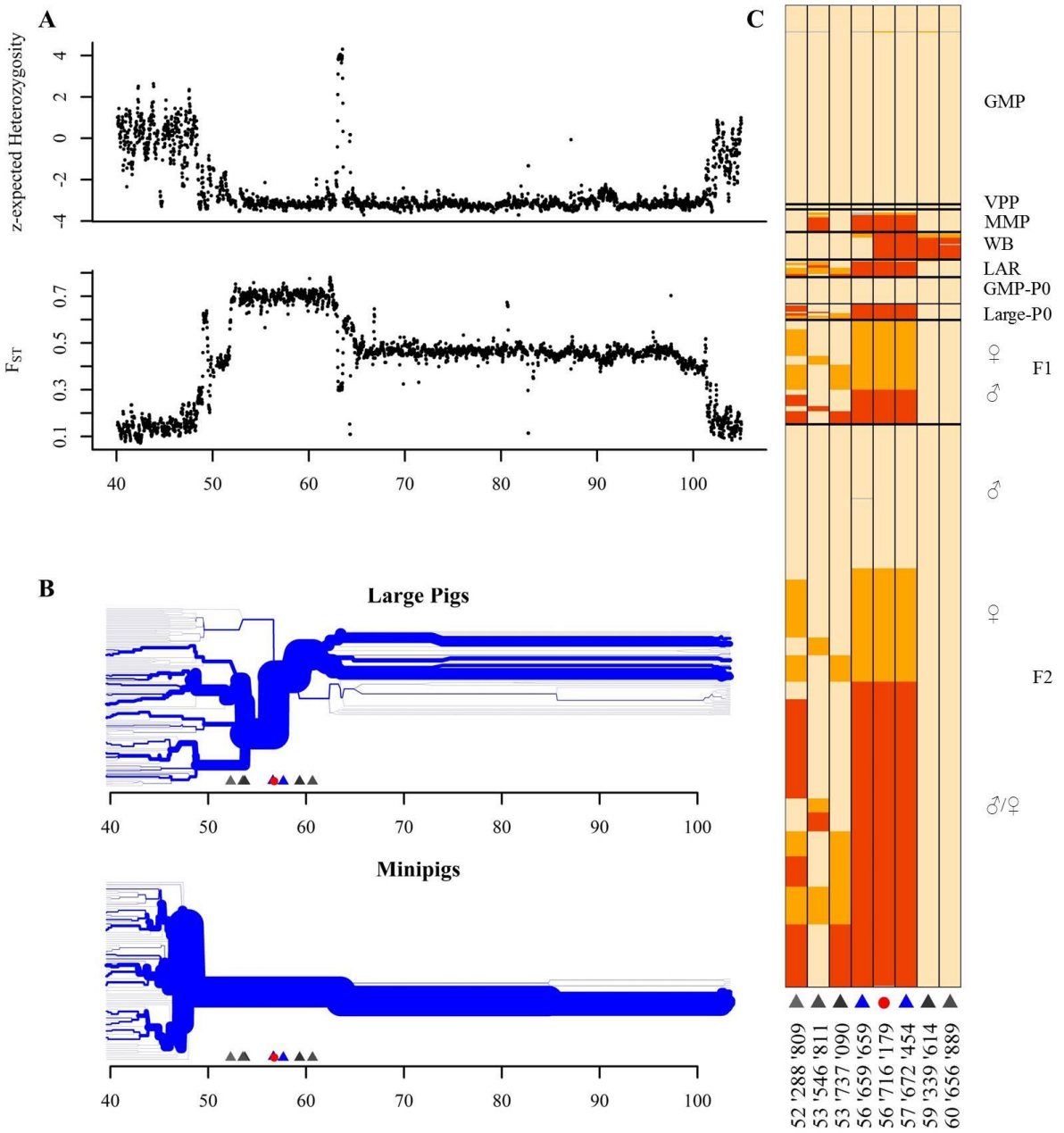

Figure 3.3: Large X-chromosomal sweep region, linkage decay and co-located genotypes in cross-bred animals. A: Normalized expected heterozygosity and fixation index between minipigs and large pigs across in the critical region of Chromosome X; B: Haplotype breakdown within the major sweep region in all large pig breeds and in the minipig breed respectively, positions in Mb, centered at 56'716'179 Mb; C: Allelic state at 8 analyzed SNPs in the sweep region between 50 and 62 Mb (red = homozygous for minipig allele, orange = hemi-/ heterozygous, beige = homozygous for opposite allele), positions in bp. Red dot and blue and grey triangles indicate SNP positions.

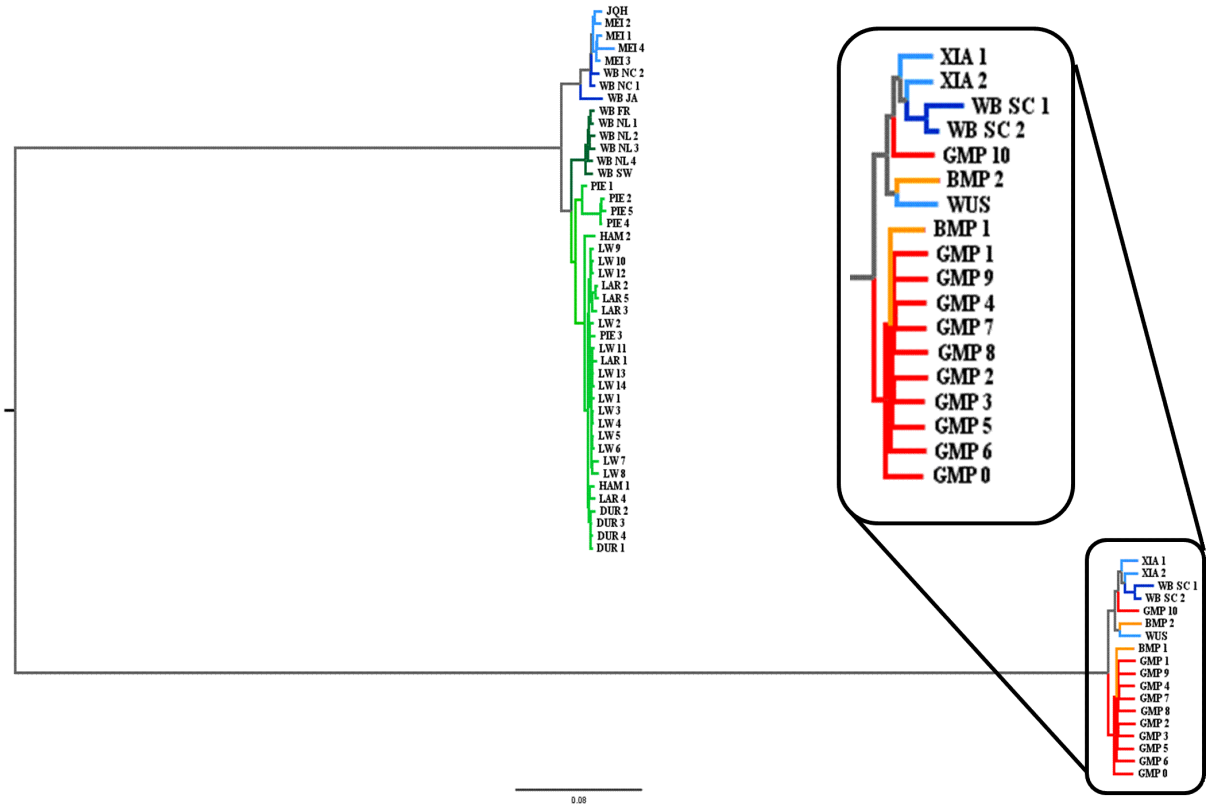

Figure 3.4: Neighbor-joining tree for all markers between 52 and 61 Mb on chromosome X. Asian wild boars in dark blue, Asian domestics in light blue, European wild boars in dark green, European domestics in light green, Mini-LEWE in orange and Goettingen Minipigs in red.

bred animals. The genotypes at the 3 center loci perfectly coincide with the affiliation of a pig to the large pigs or the minipigs, respectively (**Figure 3C**). We only observed heterozygous genotypes in animals from the cross-breeding experiment. Thus, these markers are fully informative to decide whether a cross-bred animal carries the large pig haplotype or the Minipig (South Asian) haplotype. The two markers at the end of the interval are homozygous in most European wild boars. Omitting the markers in the beginning of the interval, there are only three clearly distinguishable haplotypes within the sampled breeds in the first region of the selective sweep. **Figure 3B** shows the LD decay, depicted as a bifurcation diagram centered at position 56’716’179 for both, the large pig haplotype, based on all SNP array genotypes of all large pigs without wild boars and the minipig derived haplotype without Minnesota Minipigs. The minipig derived haplotype is stable over the whole first part of the selective sweep and is barely variable in the second part. The large pig haplotype is less stable and it splits up within the borders of the first sweep region and in the beginning of the second sweep region. The distribution of the haplotypes can be found in **Supplementary table 3.6**.

Inheritance of the haplotypes in cross-bred animals

Under the assumption of no recombination within the selective sweep region on X and the cross-breeding scheme of Pant et al. (2015), we expected a certain distribution of combinations of these haplotypes in animals of the F_1 and F_2 generation. Using the aforementioned SNP loci, we determined which haplotypes were inherited. As shown in **Table 3.3,** all F_1 females should be heterozygous and all males hemizygous for the large pig haplotype. In the F_2, half of the females are expected to be homozygous for the large pig haplotype, the other half heterozygous. The F_2 males should be hemizygous, one half for the minipig haplotype, the other half for the large pig haplotype. The observed haplotypes match the expected Mendelian proportions.

Table 3.3: Theoretical inheritance of the two segregating haplotypes on the X-chromosomes in the cross-bred pigs. Capital and low case letters indicate whether a haplotype is originating from a large pig or a minipig founder animal, respectively. Numbers of animals with the respective haplotype constellation are shown in columns right of each haplotype.

F_1	♂x	n	♂y	n	**F_2**	♂X	n	♂y	n
♀X	♀Xx	28	♂Xy	55	♀x	♀xX	90	♂xy	114
♀X	♀Xx		♂Xy		♀X	♀XX	129	♂Xy	114

Effect estimators of linear models

The distribution of phenotypic values of the analyzed traits height and length at the ages of scanning and slaughtering are displayed in **Table 3.4**.

Table 3.4: Sample size, average age, means and standard deviations for the analyzed traits in F_2 cross-breds.

Trait	N	Age [days]	Mean [cm]	SD [cm]
Height at scanning	432	63 (45-166)	39.93 ± 0.21	4.39
Height at slaughter	263	242 (166-439)	65.30 ± 0.31	5.05
Length at scanning	432	63 (45-166)	48.56 ± 0.28	5.91
Length at slaughter	410	242 (166-439)	84.16 ± 0.31	6.21

Table 3.5 shows the covariates considered in the final models for the analysis of the different traits. All non-significant higher interactions were removed from the model. We could not find a significant influence of the haplotype on the length at age of scanning and height at age of scanning, although in the latter, the p-value was 0.0718 and the subsequent conservative LSD test showed significant differences between the haplotypes. Only the sex and the age were important for length at age of scanning. The breed of the mother in the P_0 did not influence the size traits of young animals at age of scanning. **Figure 3.5** shows the estimated effects of the inherited X-chromosomal haplotype on the traits "height at slaughter" and "length at slaughter".

Table 3.5: Factors with significant influence on growth traits.

Trait	Breed	Sex	Age	Age^2	Haplotype(Sex)	Breed* sex	Breed*Age	$Breed*Age^2$
Length at age of scanning		0.003	0.016	<0.0001				
Height at age of scanning		0.29	0.057	<0.0001	0.072			
Length at age of slaughter	0.102	0.004	0.008		0.0004	0.003	0.060	
Height at age of slaughter	0.038	0.005	0.593	0.543	0.0014		0.031	0.025

For the two traits, where the haplotype effect was significant, males carrying an X-chromosome copy inherited from the minipig ancestor were significantly smaller than the ones carrying the large pig copy, while there was no significant size difference between homozygous females of large pig origin and the heterozygous females.

The respective violin plots of the linear predictors, which can be interpreted as corrected phenotypes for the four haplotype*sex classes, show a clear distinction of the males by haplotype origin.

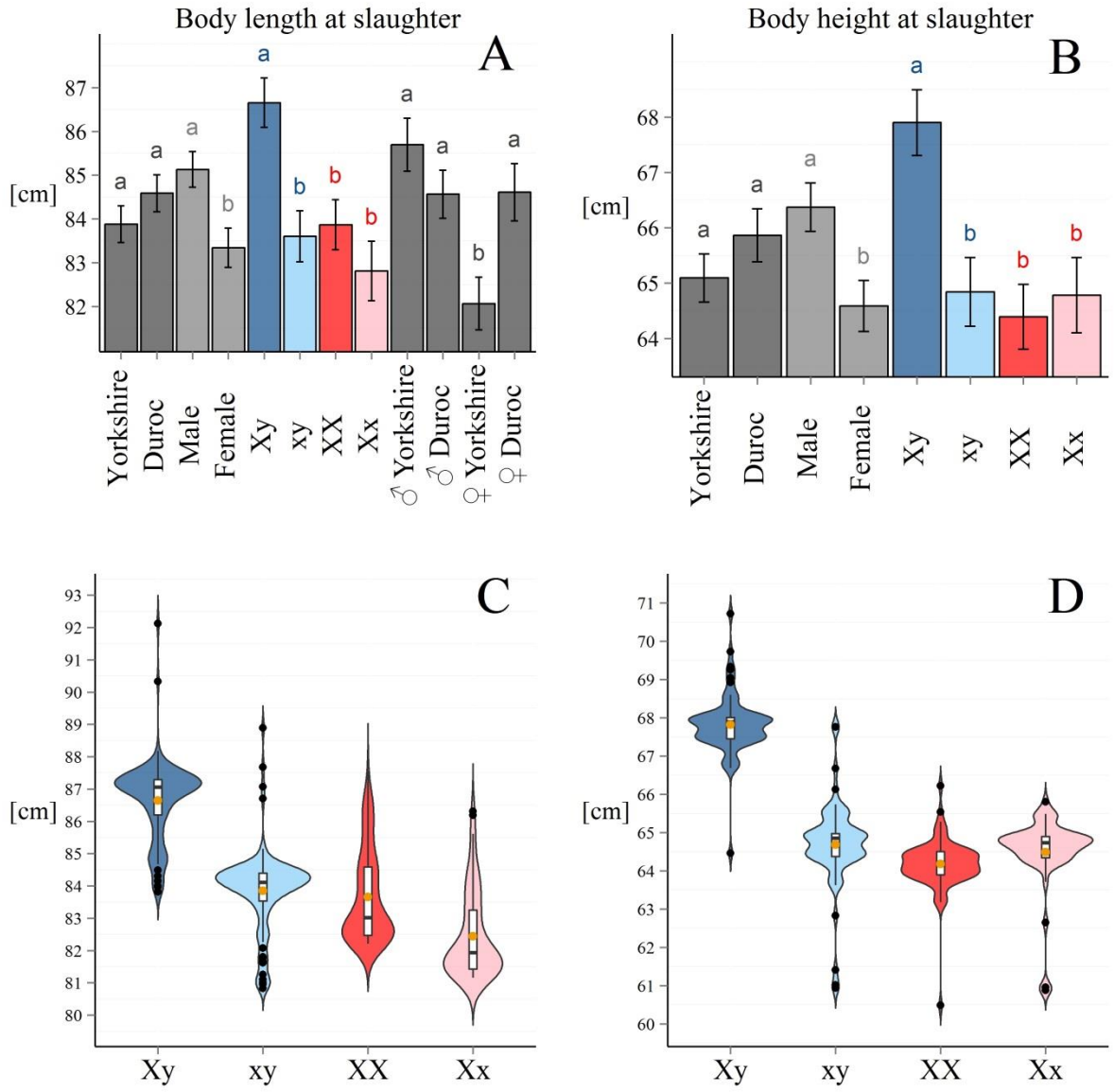

Figure 3.5: Estimated effects of the X-chromosomal haplotype state on body size. A/B: Least-square means for the significant effects for traits length and height at slaughter. C/D: Violin plots of phenotypes, corrected for all significant covariates, apart from haplotype/ sex for the respective traits.

Genes inside the Sweep

We found 18 known genes lying within the first sweep region on chromosome X (**Supplementary table 3.7**). One of those is the androgen receptor gene *AR*, which has several functions in physiological processes related to growth, body conformation and reproduction. Besides its crucial role in spermatogenesis and male fertility (Chang et al. 2004; reviewed by Wang et al. 2009), it is involved in spinal muscle atrophy (La Spada et al. 1991), bone growth (Ornoy et al. 1994) and in the determination of body size in humans, where a

mild supply of testosterone to boys of under-average size stimulated growth and sexual development without compromising final height (Richman and Kirsch 1988). Mice with a knock-out of the *AR* suffer a late onset of obesity while being normally sensitive to insulin (Fan et al. 2005). Additionally *AR* is activated by the growth factors *IGF*, *KGF* and *EGF* in tumors (Culig et al. 1994).

Discussion

This is the first study using whole genome resequencing to discover signatures of selection for body size comparing minipigs against individual and pool data of various pig breeds. Direct comparison of contrast, made up from various pig breeds each, mainly differentiated in body size only, appeared as a powerful approach to determine the genetic background of growth and size in minipigs. The high informational density of the next-generation-sequencing data promised deeper insights as the array based approaches before.

SNP Calling

One of the often discussed issues for the quality of WGS studies is the quality of the alignment and the depth at which samples have been sequenced. The proportion of aligned reads to the current reference genome of a Duroc (Groenen et al. 2012) was roughly 90 % for GMP and 87 % for BMP, similar to the proportion we find in European and Asian domestics and confirms Frantz et al. (2013) findings when mapping the WGS data of Asian wild boars (*Sus verrucosus)* against the Duroc reference . When the *de novo* assembled GMP genome with a length of 2.44 Gb (Vamathevan et al. 2013) was mapped against the Duroc reference of 2.3 Gb, about 96 % could be placed on chromosomes. Therefore, using the Duroc reference genome to evaluate minipig genomes appears appropriate, although there is an inherent risk of missing out important parts of the genome.

The number of discovered SNPs in a genome depends on the sequence identity between the reference genome and the examined samples, which is in turn dependent on the phylogenetic distance, the variation inside the breeds and the number of individuals. Additionally, the reliability of calling SNPs and determining genotypes from WGS data is also dependent on the sequencing depth, where for example reliable calling of a homozygous (heterozygous) SNP requires 15X (30X) coverage (Sims et al. 2014). From this point of view, the coverage of all minipigs was sufficient for SNP detection, but proper genotype assignment could be improved by resequencing at higher depth.

Phylogeny

Analysis of the genetic distance and F_{ST} of the sequenced animals showed a clear differentiation between European and Asian pig breeds. This result is in agreement with the current scientific consensus, that domestication occurred independently in Europe and Asia around 9000 years ago (Giuffra et al. 2000). In contrast to European breeds which evolved straight from the wild boar (Scandura et al. 2008), the history of Asian pigs is more complex: After dispersal into the islands and Oceania, interrupted by feral states, pigs were eventually transferred to the Asian mainland (Larson et al. 2007). Later, the Chinese populations diverged into a northern and a southern strain (Ai et al. 2015). Our results confirm the gap between south Chinese (Xiang, Wuzhishan) and north Chinese domestic breeds (Jiangquhai, Meishan) but appear less clear than in Ai et al. (2015).

In the phylogenetic tree, the Goettingen and the Mini-LEWE are located between the Asian and the European cluster. Looking at the breed histories, both breeds are synthetic crosses of the Vietnamese Potbellied Pig with European breeds. In the case of the Mini-LEWE, the crossing partner was the Saddleback pig and "Deutsches veredeltes Landschwein" (comparable to Large White) (Leucht et al. 1982). The GMP was established using German Landrace and the Minnesota Minipig (Glodek and Oldigs 1981), itself a cross bred of five breeds of not completely resolved but mostly north American feral, possibly Asian origin (Dettmers 1956). This might be the reason for the BMP being closer to the European cluster than the GMP.

Signatures of selection

Polygenic effect of autosomal genes on growth

This study compares two contrasting groups in order to reveal the genetic background of the reduced body size: various large pig breeds from all over the world versus a group of two minipig breeds. Such a study design has been proven efficient before in detecting regions of differentiating selection before in chicken (Rubin et al. 2010) and pigs (Rubin et al. 2012) and has revealed comprehensive sets of candidate genes in both studies. Although it is known that low recombination rates in combination with inbreeding have the potential to produce signatures similar to selective sweeps (Bosse et al. 2012), the inclusion of two genetically distinct minipig breeds should attenuate this problem. We discovered numerous putative sweep regions containing a comprehensive gene set and a first conclusion could, therefore, be that the genetic background of size differentiation is rather polygenic than mono- or oligo

genic. This is not surprising, since it is known for other vertebrate species like humans (Lango Allen et al. 2010) and chicken (Jacobsson et al. 2005) that growth has a polygenic background. The consecutive analysis of over-representation for the respective gene ontologies provided a similar picture. A variety of ontologies reached significance, comprising ontologies with functions related to growth traits and energy metabolism, like "mitochondrion" and "positive regulation of growth". The most significantly enriched ontology was 'Z disc', referring to a structural element of the muscle. The overrepresentation of genes related to mitochondria suggests that the energy metabolism might be a key element for growth restriction in minipigs. Some of the genes in significantly enriched ontologies are known to have direct effects on growth and size development or even dwarfism: A *COMT* variant increases the risk of having children with reduced birth weight (Sata et al. 2006), knock out of *TPST2* or *PATZ1* leads to growth retardation in mice (Sasaki et al. 2007; Valentino et al. 2013).

A former study by Gaerke et al. (2014) on the same GMP stock using a 60k SNP array came to similar results. They also discovered numerous regions under putative selection comprising several genes with known effect on growth and suggested a pathway connecting *SOCS2*, *GRB10* and *IGF1* as potential cause of small body size in minipigs. This finding supported the hypothesis of Simianer and Köhn (2010), that the minipig experiences a form of pituitary dwarfism, comparable to Shetland pony and Dexter cattle, supposedly caused by a deficiency of *IGF1*. This hypotheses seems natural, since the effect of *IGF1* on growth in, for example, Pygmies (Merimee et al. 1981) is known for long. In case of a mutation in an *IGF* gene, a signature of selection would be expected around the respective gene as it was found in dogs, where small breeds carry a unique coding sequence of *IGF1* (Sutter et al. 2007). However, using WGS data, we did not observe striking signals of selection near any of the known *IGF* genes or receptor loci. This coincides with findings of Zenobi et al. (Zenobi et al. 1988) who concluded that the size difference between normal sized and minipigs is neither related to serum levels of *IGF1* or *IGF2*, nor to a missing response to or reduced secretion of growth hormones. Reduced transcription, manifested in low transcription levels of the *IGF* genes or other growth hormones, could be ruled out and alterations in the underlying genes seem unlikely. But still the insulin growth factor signaling cascade is a widely considered key mechanism for growth. Our results suggest an alternative function: A possible mechanism behind the dwarf phenotype could be a resistance of the target tissues to insulin. Symptoms of this, i.e. a hampered blood glucose clearance after insulin stimulation, which could be

facilitated by a disordered lipid metabolism (Savage et al. 2007) or an intrauterine growth restriction (Jaquet et al. 2000) have been found in a feeding trial with Goettingen Minipigs (Larsen et al. 2006). Focusing on the breeds used in the cross-breeding for GMPs, the Vietnamese Potbellied Pig was the smallest, but also the most obese one (Glodek and Oldigs 1981). Even after generations of closed breeding, the major part of the GMP genome can be attributed to the VPP (Gaerke et al. 2014), suspected to be the origin for the genetically determined tendency to obesity of current GMPs. The detected signatures of selection contained genes either directly influencing insulin resistance or traits such as obesity or muscle fiber composition. Among these genes *PPARG* is an outstanding candidate, having direct effects on insulin resistance (Hevener et al. 2003) and muscle fibers (Crooks et al. 2014). Furthermore its effect on growth has been proven before in humans and pigs (Puig-Oliveras et al. 2014; Cecil et al. 2005)

Another kind of proportional dwarfism is caused by growth hormone (*GH*) deficiency (Baumann 1999) which resembles the phenotype of the "Laron dwarfism", that is accompanied by severe growth retardation and obesity (Laron et al. 1992). *GH* is also secreted in the pituitary gland and it was recently communicated that a knock-out of the growth hormone receptor gene *GHR* using genome editing technology led to further miniaturization of a bama minipig at 15 kg maturity weight (Cyranoski 2015). However, focusing on genes belonging to *GH* or its receptor genes, we find only the CLR test to show increased evidence of selection about 1 Mb away from *GHR*, but no sign of differentiation between the large and the minipig group. Therefore our results do not support the hypothesis that selection on one of the *GH* genes is underlying the minipig dwarfism.

Several genes within signatures of selection that were also comprised in significant GO terms influence *TGFB* and *FGF*, which have a known influence on growth (Stuhlmeier and Pollaschek 2004; Eguchi et al. 2001). Both are known to be involved in the mitogen activated protein kinase pathway (MAPK) that controls cell proliferation and differentiation. Klingseisen and Jackson (Klingseisen and Jackson 2011) report that this pathway plays a prominent role in growth processes and in the primordial dwarfism. This form of dwarfism leads to a proportional growth restriction causing a phenotype similar to the pituitary dwarfism. We found the central gene *MAPK1* of the Ras/ MAPK pathway in one of the largest sweep regions and other pathway genes, i.e. *PTPRR* and *MAGOH*, which hamper the MAPK signaling cascade, to lie inside strong selective sweeps. Other genes found to be under putative selection, i.e. *ACOT4*, *ATG7*, *COL7A1* and *ACACB*, interact or are directly

influenced by *TGFB* and *FGF*. The MAPK pathway gene *MAPKAPK3*, located in a large sweep on chromosome 13, is known to be involved in the mediation of growth inhibiting signals (Mayer et al. 2001) and has been found differentially expressed in the pituitary gland between the large and miniature strain of the Diannan pig (Yonggang 2010). Hence, these genes are likely to be involved in the minipig growth processes and make the MAPK-pathway a strong candidate contributing to the growth restriction in minipigs.

Major effect of the X chromosomal sweep

The porcine X-chromosome carries a selective sweep of outstanding extent (Rubin et al. 2012). Using the Chinese Wuzhishan genome reference, Ai et al. (2015) located this region of 48 Mb within the borders of 44 to 91.5 Mb, which corresponds to the region 52 to 100 Mb on the Duroc reference that we identified as a selective sweep exhibiting low expected heterozygosity in minipigs. We conclude from the same size of the region, the inclusion of partly the same samples in both studies and the nearly completely conserved haplotypes in our SNP chip analysis, that these two regions are analogous to each other. A sweep of comparable physical size was not found in recent selection signature studies in horse (Petersen et al. 2013), sheep (Kijas et al. 2012), chicken (Rubin et al. 2010), dogs (Axelsson et al. 2013) or rabbits (Carneiro et al. 2014), suggesting that this region might carry vital genetic variations kept together due to haplotype effects or that recombination in the region is suppressed. Ai et al. (2015) found a recombination breakpoint between a 14 Mb and a 34 Mb stretch, leading to three major groups of haplotypes, a European, a Southern Chinese and a Northern Chinese recombined haplotype. They explained the high differentiation of these three haplotypes with an introgression from a common ancestor even before domestication followed by a strong selective pressure for habitats in high altitudes. They concluded that this large region remained consistent over long time, since the estimated low recombination rate in this region could facilitate larger sweeps (Nachman 2001). They speculated that the reason for decreased variation was an enrichment of poly(T) sequences leading to a reduced recombination rate as known from human genomes (Kong et al. 2002). Using the Duroc reference for the analysis of our resequencing data, we find that the minipigs might carry a recombined haplotype different from the Asian and European samples we employed. This haplotype could be identified as the southern Chinese haplotype, based on the Wuzhishan samples considered in both studies. The SNP chip data within the first sweep region (52-61 Mb) shows that the founder breeds must have provided both the European and the South Asian haplotype into the GMP during breed establishment: The Vietnamese Potbellied Pig carries the South Chinese

haplotype, while the Landrace carries the haplotype found in European wild boars and the Minnesota Minipigs carries both haplotypes. Thus it is surprising that we can solely detect the South Chinese haplotype in our current GMP stock, suggesting that the European haplotype must have disappeared during breed consolidation. Since the GMP was always selected for small size and high fertility, these two traits might underlie the selection against the European haplotype.

The subsequent evaluation of an F_2 generation from a GMP x Yorkshire and a GMP x Duroc cross for four body size traits showed that males inheriting the GMP haplotype were significantly smaller than a male carrying the European haplotype for three of the four traits, while there was no significant effect on the fourth trait (Length at scanning). These results confirm that the analyzed region carries an allele influencing body size. Due to the cross-breeding scheme no females carrying only the minipig haplotypes on both chromosomes were available. The lack of a significant differentiation between females carrying the large pig haplotype on both copies of the chromosome X and heterozygous females indicates that the large pig haplotype could carry an allele that is dominant over the allele of the minipig haplotype covering the effect of the GMP allele, even though another study (Trakooljul 2004) found that the respective minipig allele of the androgen receptor AR, located in this haplotype, was dominant over a Duroc derived copy. It also could be due to the mosaic nature of the X-chromosomal activation pattern in female eutherians (Payer and Lee 2008). At the single cell level, half of the body cells are deemed to carry either an active copy of the large haplotype or the GMP haplotype. Therefore, cells carrying the large pig haplotype might attenuate the size decreasing effect of the cells carrying the GMP copy.

The differences of 3.5 % (3 cm) in body length at the age of slaughtering and 4.4 % (3 cm) in height at age of slaughtering are QTL effects of considerable magnitude. Reviewing other QTL studies on size, height and growth shows that the underlying QTL architecture can be highly different dependent on trait or organism. Whereas, in humans, height is a highly heritable trait, influenced by at minimum 180 genetic loci (Lango Allen et al. 2010) and SNP effects explain up to 45 % of the phenotypic variance (Yang et al. 2010), only a small portion could be attributed to QTLs. Gudbjartsson et al. (2008) identified 27 QTL explaining only 3.7 % of the population variance in height, composed of single effects of about 0.3 to 0.6 cm, which was confirmed by other studies (Visscher 2008: 0.4 to 0.8 cm average effect size for a QTL; Hirschhorn and Lettre 2009: 0.3 to 0.6 cm effect on adult height). In domestic animals, larger QTL effects have been found. Signer-Hasler et al. (2012) reported that two QTL

together explain 18.2 % of the heritable genetic variation in horses (~0.5 cm and ~1 cm for height at withers, respectively). They suggest the higher efficiency of QTL studies in domestic animals compared to humans to be due to the existence of isolated populations with reduced heterogeneity. In a cattle cross breeding scheme, a QTL next to *PLAG1*, *CHCHD7* and *MOS* was found with an allele substitution effect of 2 cm height at withers (Karim et al. 2011). Rubin et al. (2012) found that genotype combinations at two loci, *LCORL* and *PLAG1*, together explained a difference of 5.3 cm in body length in domestic pigs. Since we could not find signals of selection neither at *LCORL* nor *PLAG1* in our study, it is noticeable that the effect size of the chrX locus described herein has a similar effect size. Among the genes located in this region, the androgen receptor appears to be the most prominent candidate for a gene underlying the growth differences between pigs carrying opposite haplotypes, since *AR* is influenced by several growth factors (Culig et al. 1994), has known function in growth processes (Ornoy et al. 1994; Richman and Kirsch 1988) and underlies the obesity phenotype that is commonly found in minipigs (Fan et al. 2005; Johansen et al. 2001). Another study on the effect of the *AR* (Trakooljul et al. 2004) on performance and carcass traits based on a cross-breeding experiment made up with Duroc and MiniLEWE also found that Duroc and MiniLEWE carry different copies of the *AR*. It could be shown that the MiniLEWE allele led to higher expression of the *AR* in several tissues including the uterus, and had effect on several performance and carcass traits. The haplotype that contains *AR* carried by all studied GMP was most likely identical to the aforementioned MiniLEWE allele and introduced by the Vietnamese Potbellied Pig during breed foundation. This pig breed was originally not only chosen for its small size, but also for the much higher litter size compared to the Minnesota Minipig (Glodek and Oldigs 1981). Since there is a correlation between body size and litter size in mammals (Tuomi 1980), which Ferguson et al. (1984) estimated to be $r = 0.2$ in pigs, the current breeding scheme for low body weight and high fertility might have favored the Asian haplotype and *AR* could be one of the underlying causal genes.

Conclusion

Comparison of WGS data of minipigs against data of various large pig breeds is a logical approach in order to reveal the genetic background of body size in pigs. Signature of selection analysis with multiple complementary methods provided a comprehensive set of putative sweep regions, spanning approximately 2 % of the autosomal genome. The set of associated genes and the consecutive GO term overrepresentation analysis suggest that energy metabolism, alterations in the MAPK pathway, and a possible insulin resistance might be key

elements in body size inheritance of miniature pigs. Additionally, the density of resequencing data proved to be especially useful to analyze a large sweep region on chromosome X, since the SNP chip available so far holds just few SNPs of limited information in that region. We identified three SNPs on the genotyping chip, serving as perfect markers to determine the respective haplotypic state of an individual in future studies. The effect size of the QTL of 3 cm in body length and height underlying this selective sweep is comparable to the largest QTL for body size traits known from other studies in mammals. It, therefore promises interesting implications even for practical breeding.

Methods

Analysis of whole genome resequencing data

Samples and raw data preparation

We extracted DNA from 10 representative contemporary GMP sows from the experimental herd of the University of Goettingen. DNA from 2 Mini-LEWE (BMP) sows, a miniature breed developed in Berlin, Germany and a DNA-pool of 10 female BMPs from the University of Veterinary Medicine Hannover was also prepared. Whole genome re-sequencing was performed at the Science for Life laboratory at Uppsala University, Sweden on an Illumina HighSeq2000 as paired end sequencing with an aim average sequencing depth of 12X. The raw sequencing data is deposited in ENA under project accession (to be added).

We added Publicly available re-sequencing data underlying the studies of Rubin et al. (2012), Fang et al. (2012) and Vamathevan et al. (2013). These samples contained breeds of Asian and European origin, both domestic and wild, and comprised animals of the breeds Duroc (DUR), Hampshire (HAM), Jiangquhai (JQH), Large White (LW), Landrace (LAR), Meishan (MEI), Pietrain (PIE), Xiang (XIA), European wild boars (WB FR, WB NL, WB SW), Asian wild boars (WB SC, WB NC, WB JA), a Wuzhishan (WUS) and one Goettingen Minipig (GMP) (**Supplementary table 3.8**).

We aligned all sequence reads to the reference genome susScrofa3 (build 10.2;Groenen et al. 2012) using the Burrows-Wheeler algorithm as implemented in the software bwa (Li and Durbin 2009). The read trimming parameter was set to q=5. We then sorted the alignments with Samtools (Li et al. 2009) and used Picard tools (Picard 2009) to mark duplicates without removal, to down-sample the data of the single Goettingen Minipig to a coverage comparable to the other minipig individuals and to construct indices for the alignments. Single Nucleotide

Polymorphisms (SNPs) were called with GATKs Unified Genotyper (DePristo et al. 2011; McKenna et al. 2010).

In order to obtain a reliable dataset for the selective sweep analysis, we applied a stringent filtering process on the variant call set, by first removing InDels and multi-allelic SNPs and filtering with GATK for a comprehensive set of quality criteria described in the methods section. The filters for chromosome X were adjusted separately taking into account the reduced depth of this chromosome in males. In addition, to keep a sample record a minimum genotype quality (GQ) of 20 was required for sequenced individuals and a minimum depth of coverage of 4 was required for pools.

In-silico pooling

For further analyses we constructed two contrasting *in-silico* pools: the large pig virtual pool (LPP) made up of Duroc, Hampshire, Jiangquhai, Large White, Landrace, Meishan, Pietrain, the European wild boars and the Asian wild boars. The minipig *in-silico* pool (MPP) comprised the Goettingen Minipigs, the Mini-LEWE and the Mini-LEWE-pool. For this, we calculated the reference allele frequency per breed for each locus. For each called SNP, the reference allele frequency in each *in-silico* pool was then calculated as the unweighted average of the respective breed reference allele frequencies. SNP loci for which less than 50 % of the breeds in one of the two groups had a record were excluded.

Selection signature detection

For the detection of genomic regions with influence on the small size of the minipigs, we calculated heterozygosity and F_{ST} with custom R scripts (R Core Team 2015) and combined it with the composite likelihood ratio (CLR) test, implemented in SweepFinder (Nielsen et al. 2005).

We calculated expected heterozygosity per locus as $H_{exp} = 2p(1-p)$ where p is the reference allele frequency in the MPP and afterwards averaged it in sliding windows of 100 kb with 80 % overlap. We then normalized the H_{exp}values of individual windows into Z-scores by adjusting the value using the mean and standard deviation derived from all 100 kb windows along autosomes and the X-chromosome independently. We defined candidate selective sweeps using an outlier approach whereby a window that fell below a value of -2.34 (lowest 1 %) was required to initially call a sweep, such sweeps were then extended to each side until values exceeded -1.64 (lowest 5 %).

The CLR test (Nielsen et al. 2005), implemented in SweepFinder was applied to the same 100 kb windows of the filtered SNP data of all individuals belonging to the Goettingen Minipigs and the Mini-LEWE. We excluded invariable loci across both groups and took the highest 1 % of the signals further analysis.

The differentiation between the LPP and the MPP was determined by the fixation index

$$F_{ST} = \frac{\sum n_i(p_i-\bar{p})^2/(2\overline{n})}{\bar{p}(1-\bar{p})}$$

altered after Weir (1996), where p_i is the frequency of the reference allele in group *i*, $\bar{p}$ is the weighted mean frequency of the first allele in both groups, n_i is the number of samples within a group *i*, and $\bar{n}$ is the average group size. We averaged the values across the same windows of 100 kb with 80% overlap as for heterozygosity and detected regions of increased differentiation by the same method as used for expected heterozygosity, with the highest 1 % and 5 % of the actual values taken as thresholds.

A selective sweep was assumed, when regions showing decreased expected heterozygosity in the minipig or by the composite likelihood ratio test overlapped with signals of high differentiation between the two groups. We required a minimum width of 200 kb and extended the final regions by 0.5 Mb to each side. **Figure 3.6** shows the proportions of the autosomes detected to be under putative selective pressure. The CLR test detected 1 % of the genome as putative sweeps of which 59 % intersected with outstanding F_{ST} signals. The heterozygosity signature method found 5.3 % of the genome to be under selection, ~30 % thereof (1.6 % of the genome) intersecting with extreme F_{ST} signals. 0.3 % of the whole genome was shared between CLR and heterozygosity signature. Finally, we used the union of CLR and expected Heterozygosity signals intersecting with F_{ST} for further analysis.

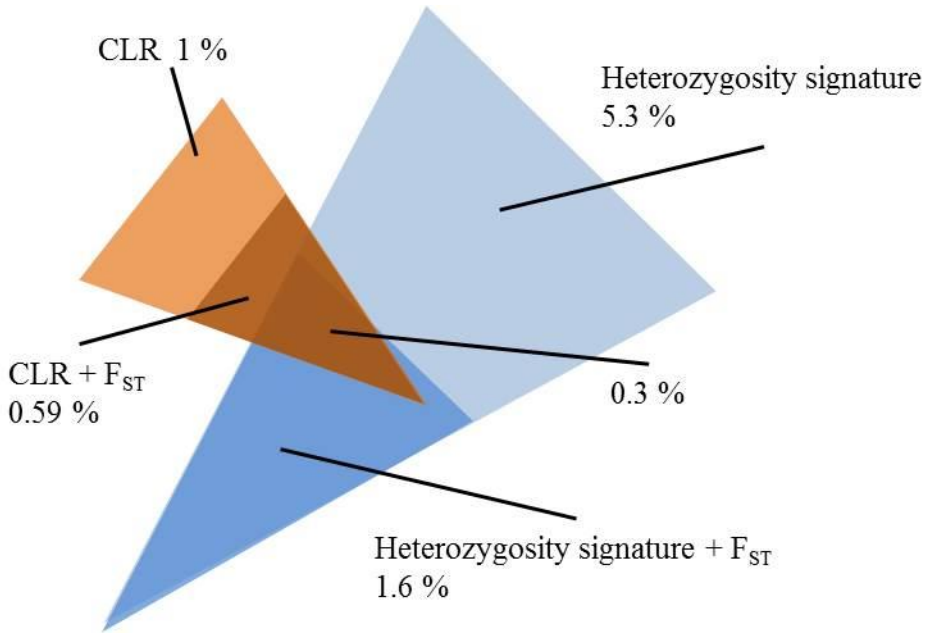

Figure 3.6: Overlaps between selection signature detection methods.

Phylogeny

We constructed phylogenetic trees from biallelic SNPs, extracted from filtered VCF-files with VCFtools (Danecek et al. 2011) and from array data with customized R-code. We calculated the pairwise distance with Plink (Purcell et al. 2007) as *1 - similarity between samples*, where similarity was the proportion of a genome of an individual being identical by state (IBS) with another animal's genome. We constructed the neighbor joining tree using PHYLIP (Felsenstein 1989), calculated Pairwise F_{ST} values from all autosomal SNP loci with 90 % or more animals with genotypes that passed filters, in each contrasting combination of the individual data of European breeds, Asian breeds and minipigs and for the subgroups European domestic breeds, European wild boars, Asian domestics, Asian wild boars, Goettingen Minipigs and Mini-LEWE, respectively (additional information on the groups can be found in **Supplementary table 3.1** and **Supplementary table 3.2**). Subsequently, we averaged values at all loci to gain a genome wide F_{ST} value.

Gene annotation and gene overrepresentation analysis

We annotated genes within regions of interest using the Ensembl Pig Gene set 79 (Cunningham et al. 2014) and, subsequently, conducted a gene ontology (GO) overrepresentation analysis by using Fisher´s exact test (Sachs and Hedderich 2006). We calculated fold enrichment *FE* as

$$FE = \frac{a}{\frac{(a+b)(a+c)}{a+b+c+d}},$$

with a being the number of genes within a sweep and the respective gene ontology, b being the number of genes within a sweep but outside the respective gene ontology, c being the number of genes in a respective gene ontology but outside a sweep and d being the number of genes outside a sweep and outside the respective gene ontology (Cramér 1945; Gene Ontology Consortium 2015). All statistics were applied on all GO terms for which genes had been found within a putative selective sweep. To account for possible bias resulting from assumption violations of the Fisher´s exact test (e.g. independency of the genes) as well as the multiple testing problem, we conducted a permutation analysis to construct empirical significance thresholds for the calculated p-values. To this end, we shifted the set of sweep regions along the genome by a random number of base pairs between 1 and the genome length, while retaining sweep sizes. Genes were then annotated to the shifted set of sweep regions and Fisher´s exact test p-value was re-estimated for each ontology term found in our original annotation. This random shifting should assure the resulting p-values to reflect the case when the null hypothesis is true. Based on 5000 replications, the 5 % quantile threshold was taken to determine the significance threshold for each gene ontology term.

Independent validation of a major sweep on the X-chromosome

For a large sweep region in the middle of chromosome X, we used additional SNP array genotype data and phenotypic data from two other studies (Gaerke et al. 2014; Pant et al. 2015) for a more comprehensive examination of this region and its effect on growth.

The samples from Gaerke et al. (2014) comprised 154 GMP, 11 MMP, 4 VPP, 16 European WB and 11 LAR. Pant et al. (2015) conducted an F2 cross-breeding experiment in which Duroc and Yorkshire females, respectively, were crossed with Goettingen Minipig males. This study provided X-chromosomal genotypes of 21 GMP males, 6 Duroc and 7 Yorkshire females, 83 F_1 animals and 454 F_2 animals. All samples were genotyped with the Illumina PorcineSNP60 BeadChip. Size phenotypes for the F_2 animals were also provided.

SNPs within the region of interest, 52 to 61 Mb on the X-chromosome were identified. We used Plink (Purcell et al. 2007) to filter out individuals with more than 90 % missing genotypes and SNPs with less than 90 % genotyping rate or a minor allele frequency of less than 1 %. Under the assumption of no recombination between the haplotypes of the European, Asian and minipig breeds we searched for loci being fixed within a group but showing

different states between groups. We then used such informative SNPs to determine the origin of the two haplotypes in the F_2 animals.

Based on the results of the sequence-based analysis, we hypothesized that the origin of the haplotype in the considered region should affect the body size of F_2 animals. We therefore classified F_2 animals in three groups: Homozygous females or hemizygous males carrying the Duroc/ Yorckshire haplotype as first class, heterozygous females as the second class and hemizygous males carrying the minipig haplotype as the third class. These classes were subsequently modeled as a fixed effect nested within sex.

Effects of the minipig haplotype on the four phenotypical traits "shoulder height at slaughter", "body length at slaughter", "shoulder height at age of scanning" and "body length at age of scanning" were estimated using proc "mixed" from SAS 9.4 (SAS 2017). The full model was

$$y_{ijk} = B_i + S_j + b_1 A_{ij} + b_2 A_{ij}^2 + H_k(S_j) + B_i \times S_j + b_3(B_i \times A_{ij}) + b_4(B_i \times A_{ij}^2) + B_i \times H_k(S_j) + b_5(A_{ij} \times H_k(S_j)) + e_{ijk}$$

where y_{ijk} is the dependent variable, B_i is the fixed effect of the breed of the female ancestor in the founder generation ($i = 1,2$), S_j is the sex, A_{ijk} is the animal's age at measurement in days, H_k is the haplotype, either 1 for homozygous females and hemizygous males carrying the large pig haplotype, 2 for heterozygous females and 3 for hemizygous males carrying the minipig haplotype. Each b_l $(l = 1,...,5)$ depicts the linear regression coefficient of the age or the respective interaction of a factor with age. e_{ijk} is the residual error. The full model was reduced by stepwise backward selection of factors with the highest p-values until only significant factors remained.

We employed the R package "rehh" (Gautier and Vitalis 2012) to estimate the extension of the two haplotypes and the decay of linkage disequilibrium around the central position of SNP 'H3GA0051810' (56'716'179 bp). Genes within this region were annotated with the Ensembl Pig Gene set 79 (Cunningham et al. 2014). Finally, QTL known from former studies located in this region were retrieved from the Pig QTL database (Hu et al. 2013, Results not shown).

List of abbreviations

BMP: Mini-LEWE; Minischwein Lehnitz-Wendefeld

Chr: Chromosome

CLR: Composite likelihood ratio test

DNA: Deoxyribonucleic acid

DUR: Duroc

ENA: European Nucleotide Archive

GATK: Genome Analysis Toolkit

GMP: Goettingen Minipig

GO: Gene ontology

GQ: Genotype quality

HAM: Hampshire

IBS: Identical by state

JQH: Jiangquhai

LAR: Landrace

LD: Linkage disequilibrium

LPP: Large pig pool

LSD: least significant difference

LW: Large White

MEI: Meishan

Mb: Mega base pairs, 10^6 base pairs

MMP: Minnesota Minipig

MPP: Minipig pool

PIE: Pietrain

QTL: Quantitative trait loci

SNP: Single nucleotide polymorphism

VCF: Variant call format

VPP: Vietnamese Potbellied Pig

WB FR: Wild boar France

WB JA: Wild boar Japan

WB NL: Wild boar Netherlands

WB NC: Wild boar North China

WB SC: Wild boar South China

WB SW: Wild boar Switzerland

WGS: Whole genome sequencing

WUS: Wuzhishan

XIA: Xiang

Declarations

Ethics approval and consent to participate

Goettingen Minipig and MiniLEWE blood samples were obtained within the course of obligatory health screening, conducted by a state approved veterinarian. No sampling was done for this study in particular.

Consent for publication

Not applicable

Availability of data

The various datasets supporting the conclusions of this article are available in the European Nucleotide Archive or Sequence Read Archive and Datadryad:

ENA accessions for FASTQ files of samples from Rubin et al. (2012): ERR173170, ERR173171, ERR173172, ERR173173, ERR173174, ERR173175, ERR173179, ERR173180, ERR173181, ERR173182, ERR173183, ERR173184, ERR173185, ERR173186, ERR173187, ERR173188, ERR173189, ERR173190, ERR173191, ERR173192, ERR173193, ERR173194, ERR173195, ERR173196, ERR173197, ERR173198, ERR173199, ERR173200, ERR173201, ERR173202, ERR173204, ERR173205, ERR173206, ERR173207, ERR173208, ERR173212, ERR173213, ERR173214, ERR173215, ERR173216, ERR173217, ERR173218, ERR173219, ERR173220, ERR173221, ERR173222, ERR173223, ERR173224; Wuzhishan Samples from Fang et al. (2012): SRR448575, SRR448588, SRR448589, SRR448591, inititially accessed through ftp://climb.genomics.cn/pub/10.5524/100001_101000/100031/reads/; SRA accessions for GMP samples from Vamathevan et al. (2013): SRR578029, SRR578191, SRR578192; ENA accessions for the GMP and Mini-LEWE data: PRJEB27654; F2 cross

breeding data from Pant et al. (2015): http://datadryad.org/resource/doi:10.5061/dryad.3jj7f; SNP data from Gaerke et al. (2014): Please send inquiries to tierzucht@agr.uni-goettingen.de.

Competing interests

The authors declare that they have no competing interests.

Author contributions

CR analyzed the data and wrote the manuscript, CR, CJR, HS designed the project and the strategy for the data analysis, CR and ARS developed the linear model used for QTL identification on the X chromosome, NTH suggested the empirical correction of the multiple testing in the GO term analysis, KHW and OD provided samples of the Mini-LEWE, SW contributed to the GMP sampling strategy, designed, financed and conducted the DNA preparation and the DNA pooling. MS developed an efficient algorithm for overlapping windows. SDP and MF provided the X-chromosome data of the cross-bred pigs. All authors discussed and reviewed the manuscript.

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Supplementary Material

Accessible through: https://figshare.com/s/228890c2f2675409d6cd

Supplementary table 3.1: Genome-wide estimated FST values between different contrasts of breed groups for all loci with call-rate ≥ 90 %, over diagonal, standard errors below diagonal

Supplementary table 3.2: Genome-wide estimated FST values between different contrasts of breed groups for all loci with call-rate ≥ 90 %, over diagonal, standard errors below diagonal

Supplementary table 3.3: Putative selective sweeps with genes contained

Supplementary table 3.4: Significantly overrepresented GO-Terms in selective sweeps

Supplementary table 3.5: Overview of SNPs in the region from 52 to 61 Mb on chromosome X, including results of filtering

Supplementary table 3.6: Occurring haplotypes in region 52 to 61 Mb on chromosome X and numbers of carrier animals

Supplementary table 3.7: Genes located in first region of large sweep on chromosome X

Supplementary table 3.8: Overview of sampled breeds and descriptive statistics of the re-sequenced samples

CHAPTER 4

Analyses of the breed integrity of the Goettingen Minipig using pool-sequencing

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Summary

The Goettingen Minipig (GMP), one of the smallest pig breeds, is an established animal model in medical research. The GMP is bred in five isolated stocks and it is of foremost importance to ensure the integrity of the different strains. We sequenced two DNA-pools from every stock and added samples from a diverse set of pig breeds from an earlier study. We estimated the pairwise fixation index for all pools, conducted principal component analyses (PCA) and functionally annotated all loci. The PCA revealed, that the GMP is easy to discriminate from all other breeds, but that there also is a certain level of differentiation between the five stocks. Annotation of all loci showed that critical functional classes, such as stop codons, were relatively underrepresented and rarely located in genes important in minipig breeding. We conclude that there is a certain level of stratification within the GMP, which might not be compromising breed integrity yet.

Keywords: Goettingen Minipig, pool sequencing, differentiation

Introduction

The Goettingen Minipig (GMP), one of the smallest pig breeds in the world, was established by crossing Minnesota Minipigs, Vietnamese Potbellied Pigs and German Landrace at the former Institute of Animal Breeding and Genetics of the University of Goettingen in the 1960s (Simianer and Köhn 2010). The university owned stock is kept at the research farm Relliehausen (RE). In 1992, a collaboration with Ellegaard Göttingen Minipigs A/S from Dalmose, DK, was started by opening unit DA1. In 2006, animals from DA2 (descendent from DA1) were brought to North Rose, NY, as foundation for a North American population. DA1 was closed down. The next separation happened in 2009 with the opening of a second barrier in Dalmose (DA3). Since the opening of the first Asian facility in Nisshin, Japan (NI) in 2013, branched off from DA3, there are now five active breeding stocks in service worldwide without exchange of animals. Even though all stocks underlie a fully documented and centrally controlled breeding scheme and are bred for the same breeding goal, the genetic isolation might harbor the risk of stratification. As the GMP is today one of the standard non-rodent animal models in medical research, its uniformity and clear characterization are of foremost importance (Bollen and Ellegaard 1997).

This study aims at identifying the traces that separation might have left in the genomes of the different populations, predict their consequences and form the base for a decision-making process as to when interchange of animals is inevitable to maintain the breed integrity.

Material and Methods

30 females representative for the respective stock were chosen for DNA sampling from every unit based on measures of pedigree-based relationship. The technically best 20 DNA extracts were randomly assigned to two groups of 10 individuals each and equimolarly pooled. Pools were sequenced at a depth of aimed 30X as paired reads on an Illumina X10. All reads were aligned to the reference genome susScr3 (build 10.2; Groenen et al. 2012) with BWA 0.7.2. (Li and Durbin 2009). The subsequent bam file preparation and variant calling followed the "GATK Best Practice" protocol (Broad Institute 2017). Due to unavailability of a high confidence learning SNP set, the 5 % SNPs with highest quality, that were also contained in dbSNP, were chosen for variant recalibration from the raw callset. Corresponding variants of various pig breeds from an earlier study (Reimer et al. 2014) were added. Individual data was virtually pooled by summation of all reference and alternative reads, respectively. Monomorphic loci were discarded, also, when a subset was used.

Reference allele frequency p_i was calculated for every pool as number of reads supporting the reference allele, divided by the total coverage at the respective locus. Wright´s fixation index was estimated pairwise (eq.1; Eding and Bennewitz 2007). A UPGMA tree, based on 100 subsamples of 50'000 SNPs ('phangorn 2.2.0'; Schliep 2011) and a principal component analysis were computed with R (R Core Team 2015). All loci were annotated with Ensembl´s variant effect predictor (McLaren et al. 2016).

$$F_{ST} = \frac{H_T - \overline{H}_S}{H_T} = \frac{\overline{p}*(1-\overline{p}) - \frac{p_1*(1-p_1)+p_2*(1-p_2)}{2}}{\overline{p}*(1-\overline{p})}, \text{ with } \overline{p} = \frac{p_1 + p_2}{2} \quad (1)$$

Results and Discussion

The UPGMA tree (**Figure 4.1**) shows that the GMP can still be considered a very distinct breed when compared to other pig breeds. Resampling shows a high robustness of the estimated tree, even when subsets of 50'000 SNPs were used.

In the PCA (**Figure 4.2**), the first principal component (PC) explains 78 % and the second 8 %. The first PC explains the variation between the GMP and all other breeds, while the second discriminates GMP from European and Asian (including Mini-LEWE) populations. It is remarkable, that the first component does not explain the difference between large pigs and minipigs, since the Mini-LEWE is also a minipig, but has a different genetic background than the GMP.

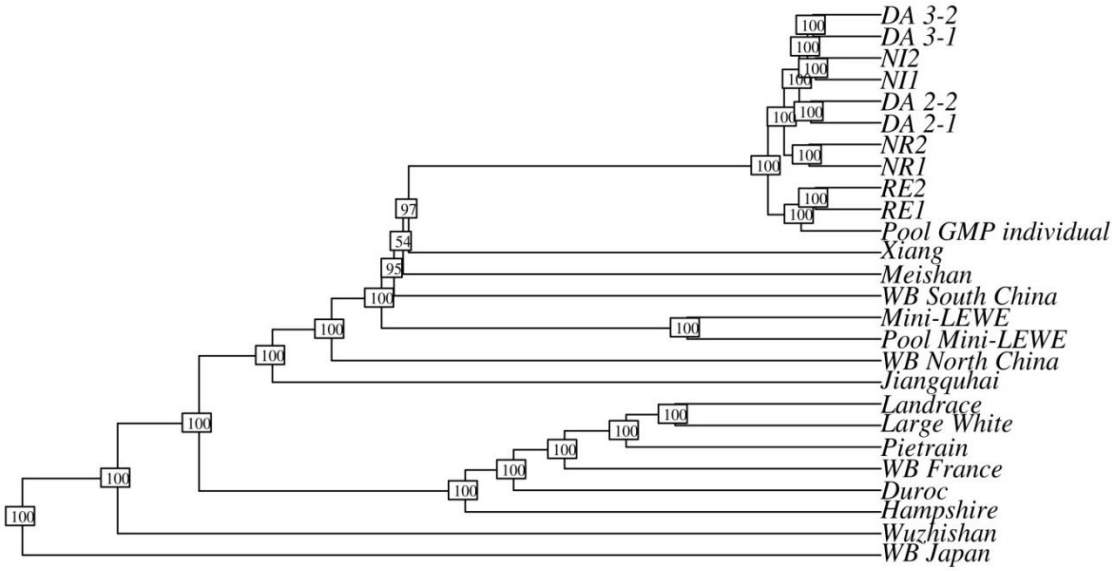

Figure 4.1: UPGMA tree of all analyzed breeds based on F_{ST}.

The PCA for the GMP pools only revealed that the DA units and the recently separated NI unit cluster together genetically. RE appears most distant from the other units, which may be explained by the long time since separation. To clarify if this led to critical functional differences, all highly differentiated SNPs were functionally annotated. In **Figure 4.3** it is shown how the relative abundance of the functional SNP classes alters along the level of differentiation.

While, for example the upstream and downstream gene variants show a steady increase towards higher F_{ST} levels, intron variants and intergenic variants remain stable throughout the entire F_{ST} spectrum. Interestingly critical classes were not represented at high differentiation, e.g. 'stop_lost' and 'start_lost', or were relatively underrepresented e.g. 'stop_gained' and 'missense variants'.

Revisiting all deleterious SNP with $F_{ST} \geq 0.9$ (**Table 4.1**), seven loci were found when NR was contrasted against another pool and one comparing RE to DA3. Among the underlying genes are annotation artefacts and novel genes, but also TMEM63A, a membrane protein gene, and PHLDA2, which has been linked to intrauterine growth restriction in humans. ZNF428, which contains the SNP differentiated between DA3 and RE has no obvious functional link to the GMP breeding goals.

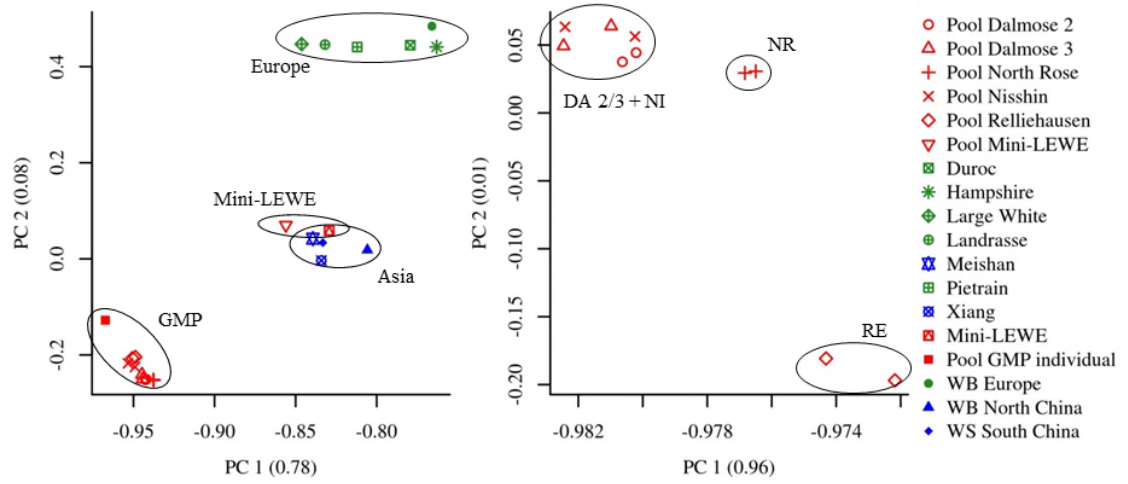

Figure 4.2: PCA based on F_{ST} of all breeds (left) and on the GMP pools only (right).

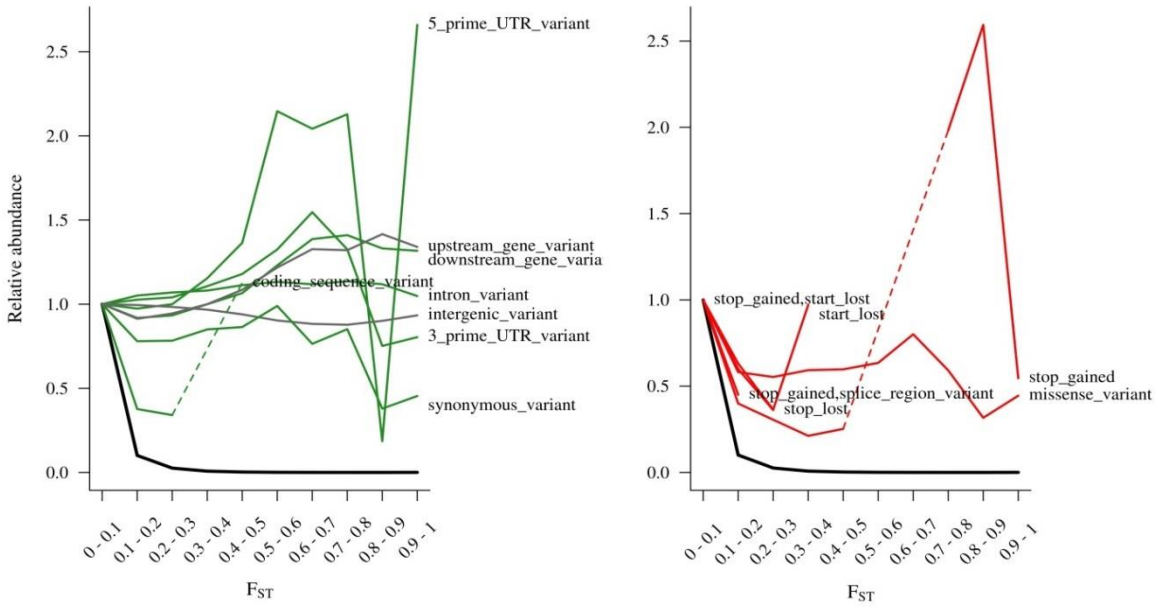

Figure 4.3: Relative abundance of selected functional classes in dependence from FST, based on the FST class 0 – 0.1.

Our results support that the GMP is still clearly distinct from all other pig breeds, but inside the GMP, differentiation between RE, NR and a cluster of NI and DA2/3 can be detected. This is sensible, since the split of NI from DA3 was just four years ago and optimal representatives of DA3 were chosen as founders of NI. The functional annotation shows that differentiation happens rather in neutral than in critical genomic regions, and differences found might rather be due to drift than to selection. The few highly differentiated deleterious SNPs are located in genes without obvious functional relation to the typical attributes of the

GMP and it seems unlikely, that they might compromise the functional integrity of the GMP. Even though genetic drift drives apart the different units genetically, the centralized breeding scheme has ensured breed integrity of the GMP so far and an exchange of animals between units does not yet appear to be necessary.

Table 4.1: Missense alleles with deleterious consequence exhibiting F_{ST} =1.

Chr	Pos	Pop1	Pop2	Ens-ID	Gene name
2	429‘370	DA2	NR	ENSSSCG00000021597	PHLDA2
2	15‘249‘414	DA2	NR	ENSSSCG00000029368	-
6	46‘206‘421	DA3	RE	ENSSSCG00000003059	ZNF428
10	16‘012‘840	DA2	NR	ENSSSCG00000010854	TMEM63A
14	7‘880‘409	DA3	NR	ENSSSCG00000025094	-
14	7‘880‘409	NR	NI	ENSSSCG00000025094	-
16	86‘466‘849	NR	NI	ENSSSCG00000020913	-
17	29‘494‘436	NR	RE	ENSSSCG00000024692	-

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CHAPTER 5

Assessing breed integrity of the Goettingen Minipig

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In preparation

Abstract

The Goettingen Minipig (GMP) is the smallest pig breed under a controlled breeding scheme and is bred in five isolated stocks. The genetic isolation harbors the risk of stratification which might compromise the identity of the breed and its usability as an animal model. We conducted whole genome sequencing of two DNA-pools per stock to assess genomic differentiation within and between stocks. F_{ST} and Reynolds distance as measures of differentiation and genetic distance were estimated for about 13M biallelic autosomal SNP loci. These data were complemented with sequence data from 13 other pig breeds from public data repositories. Based on F_{ST}, a phylogenetic tree, principal component analysis (PCA) and evaluation of functional SNP classes was conducted. An F-test was performed to reveal significantly differentiated allele frequencies between stocks, further a pathway analysis was conducted. Variation per stock was quantified as expected heterozygosity. Phylogeny and PCA showed that the GMP is easily discriminable from all other breads, but that there is also differentiation between the GMP stocks. Dependent on the contrast between GMP stocks, 4 to 14 % of all loci have significantly different allele frequencies. Functional annotation revealed that functionally non-neutral loci are less prone to differentiation which suggests, that the underlying mechanism is rather drift than selection. The pathway analysis detected differentiation between two stocks in pathway 'Glutamatergic synapse' with putative effect on behaviour. The Relliehausen stock appears to be the genetically most variable and could be a valuable resource if animal exchange is required to maintain uniformity of the GMP.

Introduction

The Goettingen Minipig (GMP) is a model organism with growing importance (Swindle et al. 2012). Bred in the 1960´s by crossing Minnesota Minipigs, Vietnamese Potbellied pigs and German Landrace, the breed is under a fully documented, closed breeding scheme ever since. The first unit was founded at the research farm of the University of Göttingen in Friedland and later resettled to the Relliehausen research farm. Due to the growing interest of customers in GMPs, this facility could not satisfy the demand anymore and therefor collaboration with Ellegaard Göttingen Minipigs A/S in Dalmose, Denmark was established in 1992. In 2003 animals from this stock were brought to Marshall BioResources, North Rose, New York as the basis of a North American GMP breeding scheme. In 2009 a second barrier was established in Dalmose, based on breeders from the first barrier, to increase the production and improve the housing conditions. With the latest transfer, animals from Dalmose were brought to a breeding unit in Nisshin, Japan in 2013. After the initial animal transfer, stocks

remained under closed breeding without any genetic exchange, albeit being under a common breeding scheme, coordinated by the animal breeding and genetics group at the University of Göttingen, Germany.

Managing the GMP in independent stocks is beneficial from a hygienic point of view. Additionally a production unit close to the main sales market minimizes negative effects on animal welfare through long transports and prevents import regulations. On the other hand splitting a population reduces the effective population size of each sub-population, which increases the risk of genetic drift or the manifestation of recessive disorders (Fitzpatrick and Evans 2009). Two concepts to counter these risks are purging of deleterious variants (Hedrick 1994) or maintenance of genetic diversity (Bosse et al. 2015). Lacy (1987) argues that drift is the most important factor in loss of genetic variance when effective population sizes are low, as in case of the GMP (Gaerke et al. 2014), and the only effective measure to mitigate adverse effects would be animal exchange.

In this study we try to assess whether the genetic management was able to maintain the uniformity of the breed GMP, or if the isolated production units are already genetically diversified such that an exchange of breeders is inevitable. This was done by re-sequencing two representative DNA pools from each unit: candidates were sampled for low average relationship within a pool, but elevated relationship towards the remaining stock, allowing an assessment of the diversity within and between units.

Material and Methods

Samples

A joint pedigree was created from the pedigrees of all five separated facilities (Relliehausen (RE); Dalmose barrier 2 and 3 (DA2, DA3); North Rose (NR); Nisshin (NI)). Numerator relationship matrices were constructed with Wrights coefficient of relationship (Wright 1922) for each stock and all animals alive within a stock in November 2015. A set of 30 individuals was selected for blood sampling with the following procedure in each facility, respectively: all candidates available for blood sampling consisting of only non-pregnant, healthy sows without genetic disorders were identified. A subset of 30 animals was randomly sampled from this list and the relationship within the set (a) and between the animals in the set and all remaining animals in the stock (b) calculated. Both values were combined in an index $I = 0.8 * a - 0.2 * b$, to minimize relationship within the samples while maximizing relationship with the sample and the remaining stock. This sampling was repeated up to

25’000 times and restarted every time a new index value went below the previously recorded one. The procedure was stopped after 25’000 rounds without improvement.

DNA of two times ten animals per stock, randomly chosen from the available samples from the previously selected 30 candidates, was pooled using equimolar amounts of the individual DNA. 150 bp paired-end sequencing was done on an Illumina HiSeq X Ten with an aim coverage of 30X and an insertsize of about 420 bp. Raw data was aligned to the reference genome susScr3 (build 10.2, Groenen et al. 2012) with BWA 0.7.12 (Li and Durbin 2009), sorting, merging of different libraries and marking duplicates were done with Picard tools 2.0.1 (Picard 2009), base qualities were recalibrated with GATKs BQSR (McKenna et al. 2010; Van der Auwera et al. 2013) using the available SNPs from dbSNP as validation (Sherry et al. 2001). Biallelic SNPs were called with the Haplotype Caller from GATK 3.4-46. SNPs were filtered with the VariantScoreRecalibration tool of GATK that uses machine learning to assess the validity of a SNP. Since there was no high quality reference set available, the 5 % SNPs with highest quality from our callset, which were also represented in the dbSNP database, were used to train the model incorporating the variant attributes QualitybyDepth (QD), MappingQuality (MQ), MQRankSumTest, ReadPositionRankSumTest, FisherStrand (FS), StrandOddsRatio(SOR) and depth (DP). A truth sensitivity filter level of 99.9 was applied.

For all loci, also represented in the study of Reimer et al. (2014, **Table 5.1**), biallelic SNP data of 13 various pig breeds (Rubin et al. 2012; Fang et al. 2012; Vamathevan et al. 2013) were added to allow inter-breed comparisons. Monomorphic loci and loci without records were removed.

Fixation index and Reynolds distance

Fixation index (F_{ST}) and Reynolds distance (D_R) were estimated between breed pools. Therefore read information of individuals was virtually pooled by breed-wise summation of reads supporting the reference and the alternative allele, respectively.

Reference allele frequency in each breed k per locus was estimated as $p_k = \frac{R_{ref}}{R_{ref} + R_{alt}}$, with $R_{ref/alt}$ depicting the number of reads supporting either the reference or alternative allele, and F_{ST} calculated per locus as $F_{ST} = \frac{H_T - \overline{H}_S}{H_T} = \frac{\overline{p}*(1-\overline{p}) - \frac{p_1*(1-p_1)+p_2*(1-p_2)}{2}}{\overline{p}*(1-\overline{p})}$, with $\overline{p} = \frac{p_1 + p_2}{2}$. Reynolds distance was estimated as $D_R = \frac{1}{2} * \frac{\sum_{i=1}^{2}(p_{1i}-p_{2i})^2}{1-\sum_{i=1}^{2} p_{1i}p_{p2i}}$, where i reflects the i^{th} allele at a

biallelic locus, namely the reference allele or the alternative allele, respectively (Eding and Bennewitz 2007). Both measures were averaged over all pairwise complete loci to gain genome-wide values.

Table 5.1: Additional porcine samples used in Reimer et al. (2014).

Breed	Number of Samples	Average Depth	Class	Subclass
Duroc	4	5.98	European	Domestic
Hampshire	2	6.49	European	Domestic
Jiangquhai	1	8.20	Asian	Domestic
Large White	14	6.46	European	Domestic
Landrace	5	6.36	European	Domestic
Meishan	4	6.83	Asian	Domestic
Pietrain	5	5.61	European	Domestic
Xiang	2	6.27	Asian	Domestic
European wild boar	6	6.44	European	Wild
Asian wild boar	5	6.27	Asian	Wild
Goettingen Minipig external	1	12.76	Minipig	Goettingen
Goettingen Minipig	10	13.01	Minipig	Goettingen
Mini-LEWE	2	13.93	Minipig	Berlin
Mini-LEWE pool	10	13.14	Minipig	Berlin
Wuzhishan	1	11.02	Asian	Domestic

Phylogeny

A phylogenetic tree was constructed from genome-wide F_{ST} values from all autosomal loci, using the clustering algorithm UPGMA as implemented in the package "phangorn" (Schliep 2011). The resulting tree reliability was determined by comparison to 100 trees constructed from 100 randomly sampled loci each.

Test of allele frequency differences between pools

We employed an F-test based statistic to determine statistically significant variation patterns between pools for every locus (eq. 1).

$$F = \frac{V_I}{V_O}, \quad (1)$$

where V_I is the pooled variance within a unit, e.g. RE1 and RE2, estimated as $V_I = \frac{p_{RE1}+p_{RE2}}{2} * \left(1 - \frac{p_{RE1}+p_{RE2}}{2}\right) * \frac{2}{10}$, and where V_O represents the variance between the aforementioned unit and a remote pool, e.g. NI1 estimated as $\frac{p_{RE1}+p_{NI2}}{2} * \left(1 - \frac{p_{RE1}+p_{NI2}}{2}\right) * \frac{2}{10}$. Degrees of freedom where assumed nine, for every pool was made up from ten animals.

Heterozygosity, fixed alleles and private polymorphisms

Expected heterozygosity at locus i was estimated from original pools and the virtual pool for each stock as $H_{exp_i} = 2 * p_i * (1 - p_i)$, where p_i is the reference allele frequency. It was further assessed whether a single stock was fixed for one allele, while the others were fixed for the other allele. To assess variability remaining in only one stock, loci where all stocks apart from one were fixed, were identified. This was done both for the subset of loci without missing information and for loci where single stocks had missing information.

Annotation

Loci identified in the aforementioned tests were functionally annotated with the Ensemble Genes database (version 89; Sscrofa 10.2; Aken et al. 2016).

Gene and pathway analyses

For each comparison, we used the SNP-wise F_{ST}-values to identify genes and pathways that are enriched with highly differentiated SNPs between the contrasts. To this end, we first created a SNP to gene annotation based on the Ensembl Genes database (version 89; Sscrofa 10.2). We then performed a Kolmogorov-Smirnov test to test whether the distribution of the F_{ST}-values within each gene significantly differs from the distribution of all the F_{ST}-values on the respective chromosome. To correct for multiple testing we permuted the gene positions and repeated the analyses with the permuted set of genes. We then used the p-values of the permutation test to obtain a genome-wide significance threshold for our p-values. Using the KEGG database (Kanehisa and Goto 2000), we subsequently annotated the Ensembl genes to KEGG pathways. In a similar fashion to the gene analyses, we performed a Kolmogorov-Smirnov test to compare the p-values of the genes in each annotated pathway to the p-values of all genes.

Results

Sampling of the optimally representative candidates for pooling based on relationship measures resulted in candidate sets which exhibited lower inner-set mean relationship

coefficients (*a*) compared to the mean relationship of the candidate set with the remaining stock mates (*b*). Both, the absolute level of relationship and the difference between *a* and *b* were lowest for RE and highest for NR, while DA2 and 3 and NI were at the same level and exhibited similar difference between a and b (**Table 5.2**).

Variant calling discovered 21'779'266 raw SNPs, with 3'185'086 SNP thereof not documented in dbSNP. After variant quality score recalibration 16'000'684 total SNPs and 937'592 novel SNPs were retained. Intersection with the data set of various pig breeds and discarding of monomorphic loci provided a set of 15'022'059 analysis ready SNPs on chromosomes 1 to 18 and X.

Table 5.2: Mean coefficients of relationship within a stock sample and between the sample and the remaining stock, including number of successfully extracted probes.

	RE	DA2	DA3	NR	NI
Relationship within sample	0.357	0.396	0.396	0.403	0.387
Relationship Sample/ Remaining Stock	0.376	0.404	0.402	0.410	0.397
Successful DNA extractions	28	24	23	28	24

Differentiation and distance measures

As measures of differentiation and genetic distance between the different breeds F_{ST} and Reynolds genetic distance (D_R) were estimated. When applied on the variable set of large breeds and minipig pools, both measures provided a similar picture of three strongly differentiated groups (**Figure 5.1**). In principle, these three groups were the minipigs, the European breeds and the Asian breeds, respectively, with the exception, that the Mini-LEWE pools clustered with the Asian group. Comparing F_{ST} against D_R, the latter showed generally higher estimates, relatively inflated at moderate levels of differentiation/ distance (**Figure 5.2**), but provided in general a very similar picture. Therefore, only F_{ST} was used for later purposes, such as the functional annotation. Focusing on the differentiation within the three groups, the GMP exhibited the lowest average differentiation (F_{ST}: 0.05; D_R: 0.09; see also **Supplementary table 5.1**), the European (F_{ST}: 0.17; D_R: 0.26) the second lowest and the Asian the highest (F_{ST}: 0.27; D_R: 0.36) (**Table 5.3**). The average differentiation to other groups was higher than the differentiation within the own group, clearly so for minipigs and for European breeds, but not as clear for the Asian breeds. These exposed an even lower

average D_R (0.34 vs 0.36) and F_{ST} (0.25 vs. 0.27) to the GMP than within the group of Asian breeds.

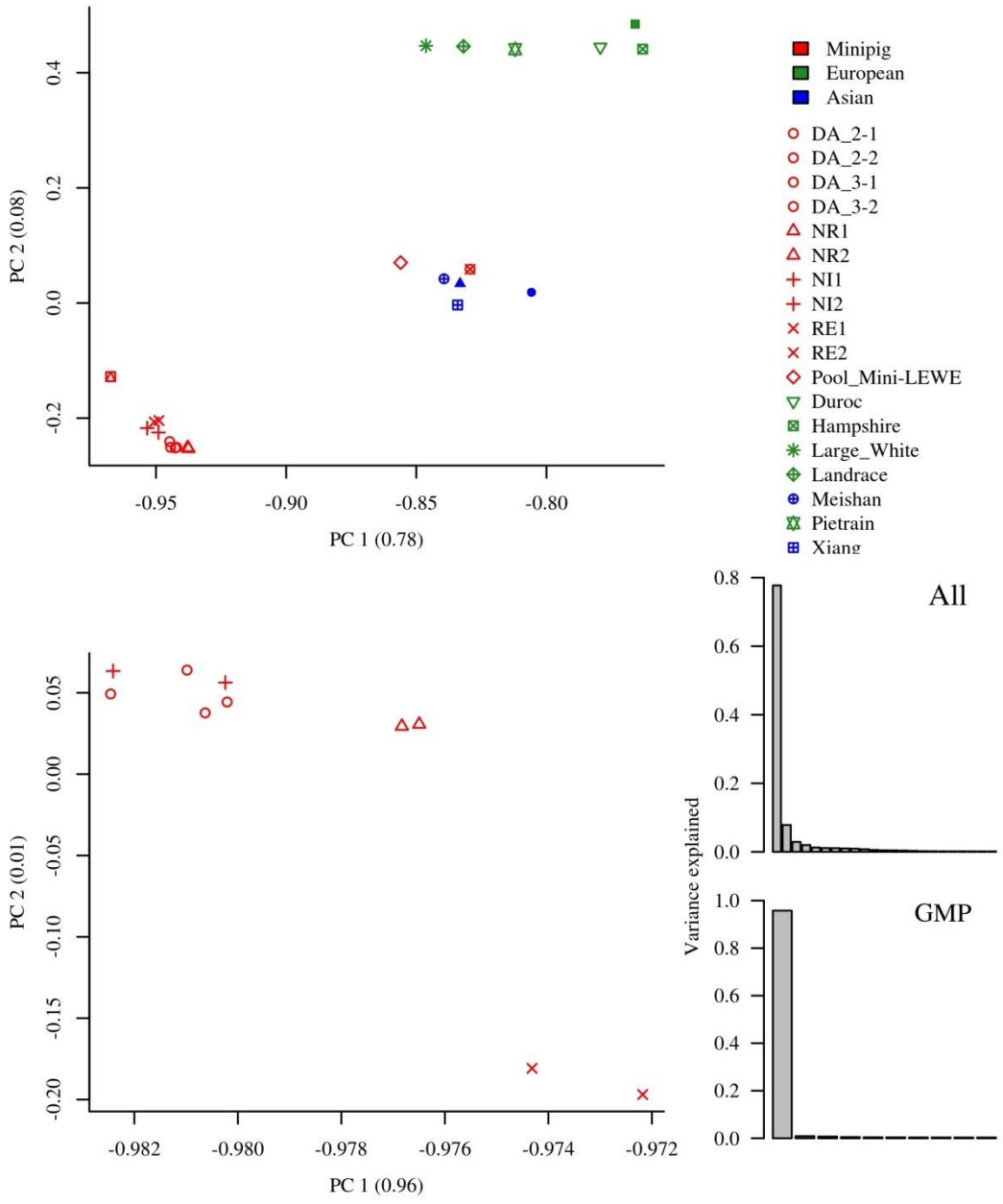

Figure 5.1: PCA of pairwise genome-wide FST including all pools (top) and GMP DNA pools only (bottom); Variance explained by PC in brackets. Distribution of PC´s on the right.

Principal component analysis of D_R and F_{ST} resembled each other with only marginal deviations ($r > 0.99$). The first component accounts for 78 % of the variation in F_{ST} values and discriminates between the GMP and the other breeds, while the second component, explaining eight percent, separates the GMP, Asian and European breeds. Analyzing the GMP

separately, the first component explains already 96 % of the variation among the GMP pools and separates the RE pools from the other stocks. The second component (1 %) explains differences between RE, NR and a group consisting of the DA and NI pools.

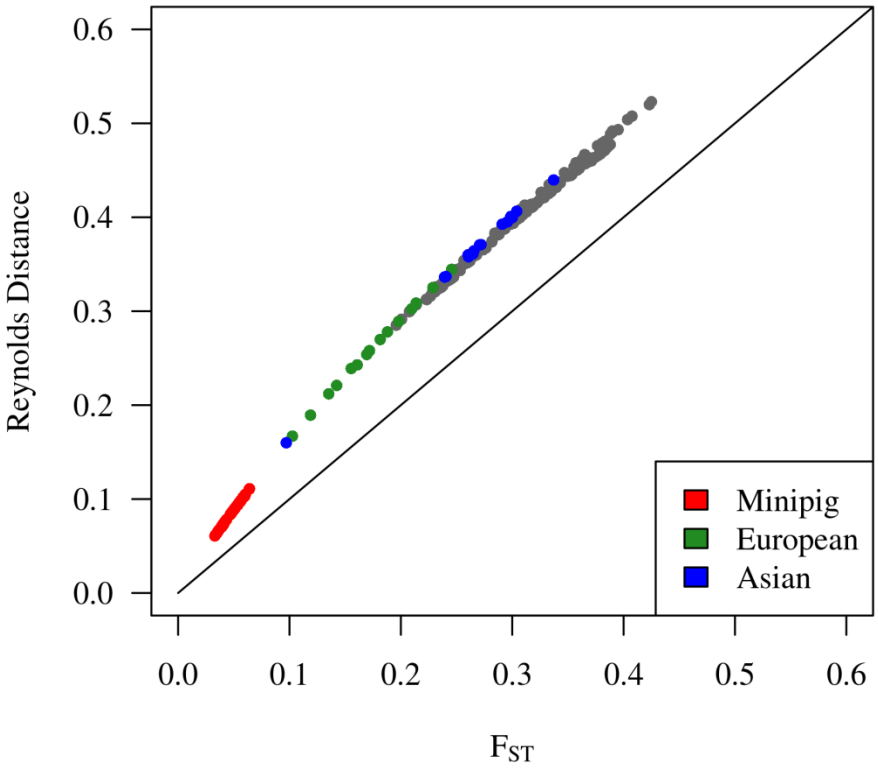

Figure 5.2: Genome wide FST vs. Reynolds distances for all pairwise comparisons. Comparisons within breed types in the respective colors, comparisons between breed types in grey.

Table 5.3: Mean F_{ST} and D_R between European and Asian breeds and GMP.

		GMP	Asian	European
FST	GMP	0.049	0.245	0.335
	Asian		0.267	0.348
	European			0.175
D_R	GMP	0.086	0.337	0.427
	Asian		0.363	0.448
	European			0.260

Phylogeny

The UPGMA tree (**Figure 5.3**) produced from F_{ST} values calculated with genome wide SNP data shows a clear clustering of the GMP from the other breeds. The next level clusters contain (in that order) Xiang, Meishan, South Chinese wild boars, the Mini-LEWE and the North Chinese wild boars. The European breeds form their own cluster. For all 100 samplings, the GMP cluster, the Mini-LEWE cluster and European cluster are rediscovered in every iteration, while the nodes connected to the Asian breeds seem unstable with resampling probability between 9 and 8 %, with the exception of the Japanese Wild boar, that behaves like an outgroup sample. Even though, the European and the GMP clusters are distinct, the order within the clusters is variable. The node support within the European cluster spans from 62 to 69 %, and between 20 to 72 % in the GMP cluster. The most stable structure with 72 % contains the RE pools and the least stable (20 %) contains the DA and NI herds.

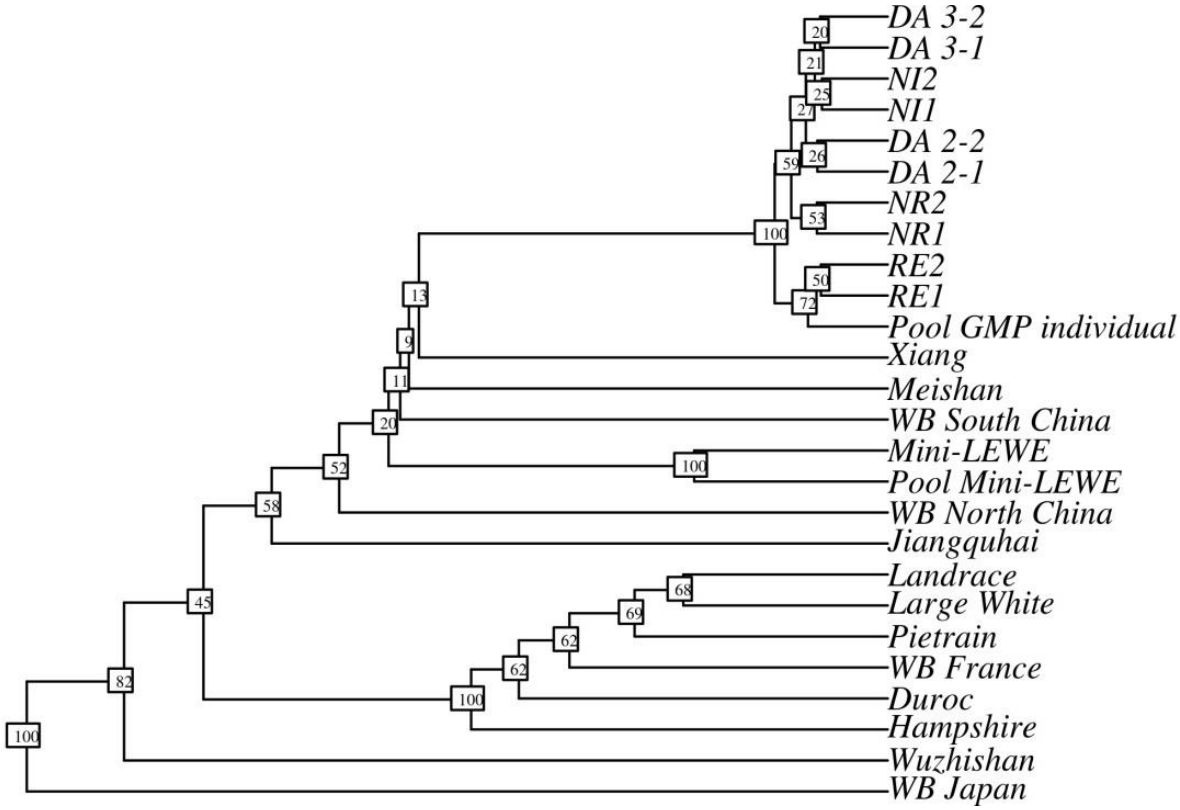

Figure 5.3: UPGMA tree based on genome-wide FST values; resampling frequency based on 100 random samples of 100 loci in rectangles.

Stratification within the GMP

The genetic differences within the GMP were determined by comparing pools in terms of allele frequency differences, such as oppositely fixed alleles, extreme F_{ST} values between stocks, differences in the average expected heterozygosity within pools by a variation based

approach employing an F-test statistic. Resulting loci detected by the aforementioned statistics were functionally annotated and imbalances between the various classes were checked for potential biases towards differentiated loci. Finally, stocks were compared by gene based and pathway based approaches.

Significance test of pool allele frequencies between and within stocks

The F-test compared the variation between the two pools with the variation between one of the pools against one foreign pool and could, in contrast to $F_{ST,}$ add probabilistic evidence on differentiation between pools. On average, the NI stock had the lowest proportion of significantly (p=0.05, Bonferroni corrected) differentiated loci, overall 4.9 %, second lowest was RE with 6.1 % followed by DA3 and 2 with 6.9 and 7.3 %, respectively. With 9.4 %, NR had the highest proportion of differentiated loci (**Table 5.4**). Focusing on the stocks separately (**Figure 5.4**), only RE had comparable amounts of differentiated loci with all others. From the perspective of DA, NI, and NR, the level of differentiation to RE was clearly highest throughout all comparisons.

Table 5.4: Proportion of SNP significantly different between stock and remote pool in F-test at 5 %.

	RE_1	RE_2	DA2_1	DA2_2	DA3_1	DA3_2	NR_1	NR_2	NI_1	NI_2
RE	0	0	0.08	0.08	0.07	0.08	0.07	0.07	0.08	0.09
DA2	0.13	0.13	0	0	0.08	0.08	0.07	0.08	0.08	0.08
DA3	0.12	0.12	0.08	0.08	0	0	0.08	0.08	0.06	0.07
NR	0.14	0.15	0.10	0.10	0.11	0.11	0	0	0.10	0.12
NI	0.10	0.09	0.05	0.05	0.04	0.04	0.06	0.05	0	0

Also, the number of evaluated loci ranged between 3.4 and 4.6 M, where the highest numbers occurred, when RE or NI were involved. Mostly both tested pools of a stock showed a similar amount of differentiation with the exception of NR versus the two NI pools. The highest proportion of differentiated loci was found, when NR was tested against the two RE pools.

Expected heterozygosity

Expected heterozygosity, as measure of variation within a pool, revealed that RE and NI are systematically more heterozygous than DA 2, DA 3 and NR (**Table 5.5**). When estimated for single pools expected heterozygosity was between 0.21 and 0.22 for DA 2 and DA 3 and NR

and between 0.24 and 0.25 in NI and RE. Estimated from the virtual union of both pools per stock, the values were about 0.03 higher, but systematic differences remained (**Table 5.6**).

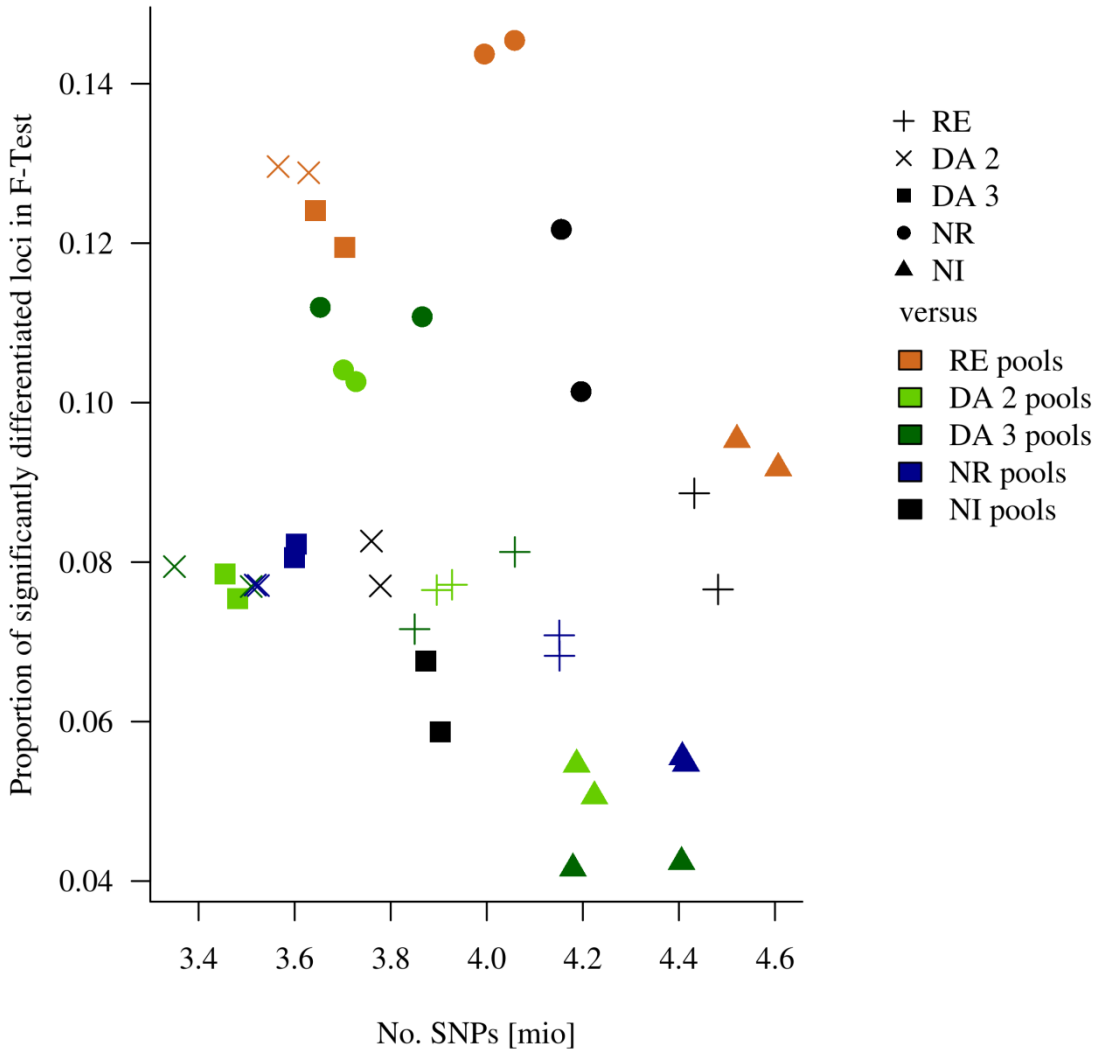

Figure 5.4: Proportion of significantly different loci at 5 % Bonferroni corrected F-test level against No. of tested loci.

Table 5.5: Expected Heterozygosity within pools.

	RE_1	RE_2	DA2_1	DA2_2	DA3_1	DA3_2	NR_1	NR_2	NI_1	NI_2
H_{exp}	0.24	0.25	0.21	0.21	0.22	0.22	0.21	0.21	0.24	0.25
SD	0.18	0.18	0.18	0.18	0.18	0.18	0.18	0.18	0.18	0.18
Nloci [M]	8.2	8.4	7.1	7.1	7.0	7.5	7.7	7.7	8.7	8.5
NNA [M]	4.5	4.3	5.6	5.6	5‘8	5.2	5.0	5.0	4.1	4.2

Table 5.6: Expected Heterozygosity estimated from the virtual union of both unit pools.

	RE	DA2	DA3	NR	NI
H_{exp}	0.27	0.24	0.24	0.24	0.27
SD	0.17	0.17	0.17	0.18	0.17
Nloci [M]	10.7	9.5	9.7	10.1	11.0
NNA [M]	2.0	3.2	3.1	2.6	1.8

Fixed alleles and private polymorphisms

Table 5.7 depicts the correlation of allele frequencies of loci that had complete recordings and where each stock was fixed for either the reference or the alternative allele. Only 554 loci fulfilled this criterion. The correlation between the stocks based on these loci was negative for pairs where RE was involved. Negative correlations ranged from -0.12 with NI to -0.30 with NR. For all other combinations correlations were positive and ranged from 0.26 (DA 2 to MA) to 0.51 (DA2 to NI). On the other hand, RE held by far the largest number of still variable loci while the other pools were fixed at one allele. Out of the 560’855 loci fulfilling the criterion of one variable stock while all others were fixed, 275’295 belonged to RE (**Table 5.8**). NR (88’580) and NI (83’402) with about 80’000 loci carried more than the DA units (59’319 and 54’259). Including loci with missing information increased the total number to 1’194’559 loci. The predominance of RE was with 461’177 loci less distinct than before. Notably NI still carried more such loci than NR (296’485 vs. 198’062).

Table 5.7: Correlation between genotypes for loci that were completely fixed within each unit.

	RE	DA2	DA3	NR	NI
RE	1	-0.22	-0.19	-0.30	-0.12
DA2	-0.22	1	0.45	0.26	0.51
DA3	-0.19	0.45	1	0.30	0.51
NR	-0.30	0.26	0.30	1	0.36
NI	-0.12	0.51	0.51	0.36	1

Table 5.8: Number of private polymorphism; left: completely recorded loci; right: missing information (NA) allowed.

	Without NA	with NA
RE	275,295	461,177
DA2	59,319	104,128
DA3	54,259	134,707
NR	88,580	198,062
NI	83,402	296,485

Annotation

Functional annotation of loci significant in F-test, showing oppositely fixed alleles and exhibiting extreme fixation index values reveled that most loci were in intergenic or intronic regions (compare **Table 5.9**, i.e. F-test: 62 % intergenic and 27 % intron) followed by 10 % upstream and downstream variants. Exonic variants were present to an extent of less than 1 %. Potential protein changing variants like start or stop codons were barely present at a 5 % significance level in the F-test and absent among loci with oppositely fixed alleles. Compared to the unselected background, intergenic, intron, up –and downstream variants were similarly represented in both, the 5 % F-test level and for the oppositely fixed loci, while missense variants were about 20 % less frequent in oppositely fixed loci and stop codons were not present at all.

Annotating SNPs in different levels of F_{ST} differentiation supported these findings. Start and stop losses could not be found at higher F_{ST} levels, missense mutations and especially stop gains showed a decline in frequency towards high F_{ST} values, while up- and downstream, intron and intergenic variants were unaffected or increased in frequency (**Figure 5.5**). In the highest F_{ST} class with values > 0.9 there were 60 missense variants in all pairwise comparisons (**Supplementary table 5.4**), 8 of which had a predicted deleterious function (**Table 5.10**). While some annotations pointed to artefacts or novel genes, 3 of them were located in genes known by name, among them *Pleckstrin homology-like domain family A member 2*(*PHLDA2,* DA2 vs NR), *Zinc Finger Protein 428* (*ZNF428,* DA3 vs RE) and *Transmembrane protein 63A* (*TMEM63A,* DA2 vs NR).

Table 5.9: Relative amount of significantly differentiated and oppositely fixed loci per functional class and relative abundance of loci in differentiated classes in comparison to all background loci.

	Relative amount of loci per class		Relative abundance compared to background	
	5 % bonf	Opp. Fixed	5 % bonf	Opp. fixed
3_prime_UTR_variant	0.3916	0.5342	1.0168	1.3002
5_prime_UTR_variant	0.0721	0.0981	1.0081	2.0146
coding_sequence_variant	0.0001	0	0.3129	0
downstream_gene_variant	5.0820	5.7021	0.9992	1.0587
intergenic_variant	61.6988	62.1784	0.9999	1.0083
intron_variant	26.9416	26.3410	0.9974	0.9898
missense_variant	0.2786	0.2617	0.9992	0.8039
start_lost	0.0006	0	1.0470	0
stop_gained	0.0030	0	0.9434	0
stop_gained,splice_region_variant	0.0001	0	0.5743	0
stop_gained,start_lost	0.0000	0	0	0
stop_lost	0.0001	0	0.9208	0
synonymous_variant	0.4815	0.3707	0.9977	0.8098
upstream_gene_variant	5.0498	4.5137	1.0169	0.8840

Table 5.10: Annotation of deleterious missense variants with pairwise FST of 1.

Chr	Pos [bp]	Pool 1	Pool 2	Ensembl ID	RS ID	SIFT	Gene name
2	429‘370	DA_2	NR_4	ENSSSCG00000021597	rs320902190	0.03	PHLDA2
2	15‘249‘414	DA_2	NR_4	ENSSSCG00000029368	-	0	-
6	46‘206‘421	DA_3	RE_1	ENSSSCG00000003059	-	0.02	ZNF428
10	16‘012‘840	DA_2	NR_4	ENSSSCG00000010854	rs792023778	0.01	TMEM63A
14	7‘880‘409	DA_3	NR_4	ENSSSCG00000025094	-	0.01	-
14	7‘880‘409	NR_4	NI_5	ENSSSCG00000025094	-	0.01	-
16	86‘466‘849	NR_4	NI_5	ENSSSCG00000020913	rs711954795	0.04	-
17	29‘494‘436	NR_4	RE_1	ENSSSCG00000024692	rs344262225	0.03	-

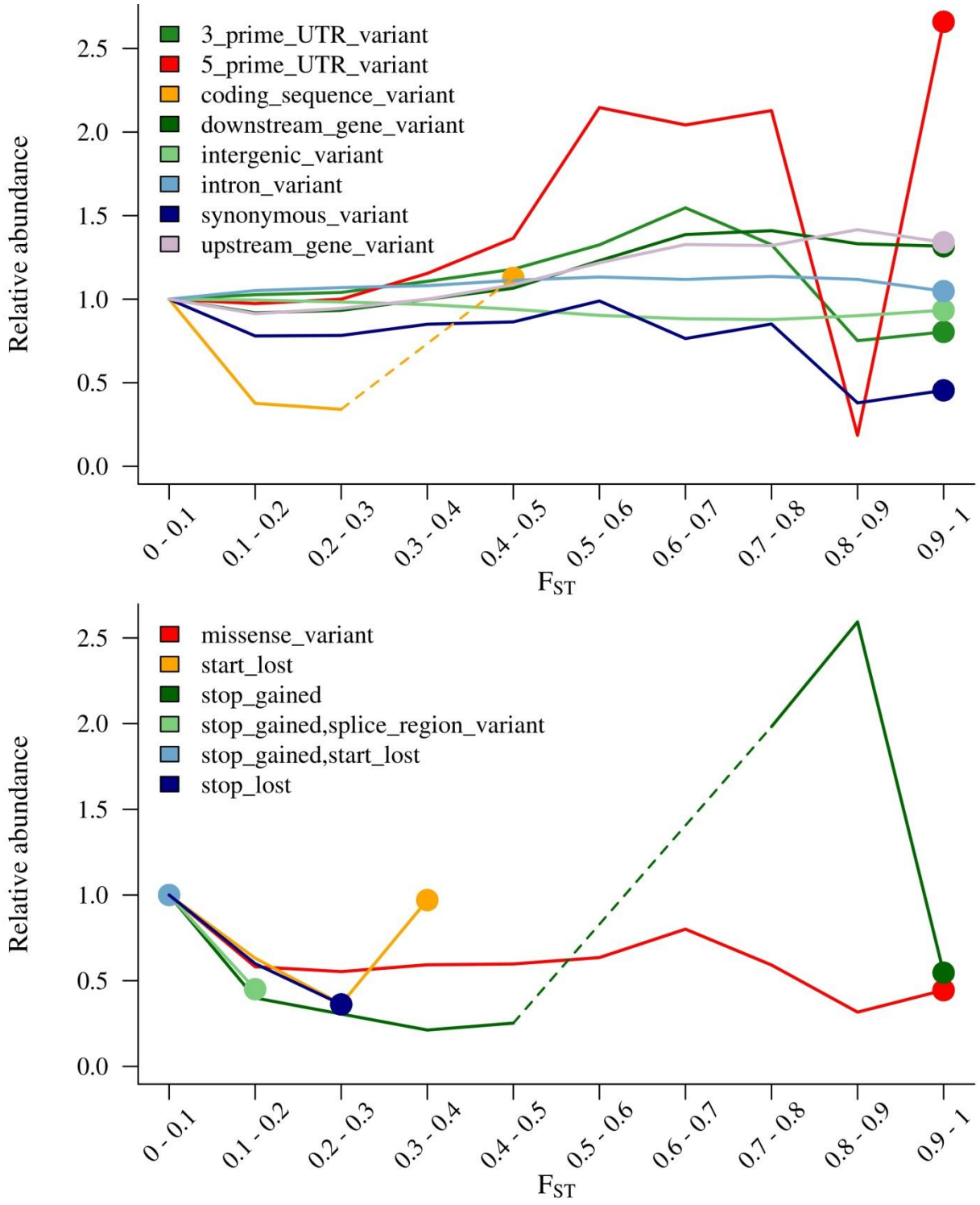

Figure 5.5: Relative abundance of functional SNP classes in dependence from pairwise FST bewteen units.

Annotation of loci with variability in only one stock, while all other stocks were fixed, resembled the fractions of functional classes already known from the F-test and F_{ST} annotations (**Table 5.11**), but due the higher number of private variable loci in RE, the absolute numbers of loci annotated to potentially protein changing classes, such as missense mutations, was therefore higher in RE (1'499) than in all other stocks. Both DA stocks carried the lowest number of missense mutations (356 and 325). Still, every stock carried at least one stop codon gain or loss, RE even 11.

Gene based and pathway tests

The Kolmogorov-Smirnov based on pairwise testing of 316 annotated KEGG pathways detected only the pathway "Glutamatergic synapse" to be differentiated between DA3 and NR at the 5 % Bonferroni corrected level. Glutamatergic synapses are involved in signal transduction, specifically neuronal excitability, in the nervous system (Bergles et al. 2000) and are vital for brain function (Siddoway et al. 2011). No single gene was found to be significantly differentiated.

Table 5.11: Relative amount (in per cent) of private polymorphism loci per functional class (absolute number of loci in brackets).

	RE	DA2	DA3	NR	NI
3_prime_UTR_variant	0.3968	0.3702	0.3451	0.3863	0.4227
	(1,085)	(218)	(186)	(340)	(350)
5_prime_UTR_variant	0.0914	0.1206	0.0853	0.1159	0.1123
	(250)	(71)	(46)	(102)	(93)
coding_sequence_variant	NA	NA	NA	NA	NA
	(NA)	(NA)	(NA)	(NA)	(NA)
downstream_gene_variant	4.3245	4.791	4.4469	4.0784	5.0805
	(11,824)	(2,821)	(2,397)	(3,590)	(4,207)
intergenic_variant	65.1176	63.968	65.1318	65.4871	62.6211
	(178,042)	(37,665)	(35,108)	(57,645)	(51,854)
intron_variant	23.8819	24.0111	23.8039	23.5092	24.5936
	(65,297)	(14,138)	(12,831)	(20,694)	(20,365)
missense_variant	0.5482	0.6046	0.6029	0.6169	0.698
	(1,499)	(356)	(325)	(543)	(578)
start_lost	0.0004	NA	NA	NA	0.0012
	(1)	(NA)	(NA)	(NA)	(1)
stop_gained	0.0037	0.0034	0.0037	0.0023	NA
	(10)	(2)	(2)	(2)	(NA)
stop_gained,splice_region_variant	NA	NA	NA	NA	0.0012
	(NA)	(NA)	(NA)	(NA)	(1)
stop_gained,start_lost	NA	NA	NA	NA	NA
	(NA)	(NA)	(NA)	(NA)	(NA)
stop_lost	NA	NA	NA	NA	NA
	(NA)	(NA)	(NA)	(NA)	(NA)
synonymous_variant	1.3682	1.6372	1.4545	1.4791	1.6533
	(3,741)	(964)	(784)	(1,302)	(1,369)
upstream_gene_variant	4.2671	4.4938	4.1259	4.3249	4.8161
	(11,667)	(2,646)	(2,224)	(3,807)	(3,988)

Discussion

The aim of our study was to identify, if the integrity of the breed Goettingen Minipig was compromised by the current production and genetic management system that relies on genetic isolation of production units. First, the classification of the GMP samples in the context of various pig breeds representing worldwide porcine genetic variation was evaluated with phylogenetic and population genetic methods. Second, genetic identity within the breed was assessed by multiple approaches describing variability within and differentiation between the separated stocks.

Discriminability of the Goettingen Minipig from other pig breeds

Our PCA results based on Reynolds distance and F_{ST} show clearly distinct groups of European pigs, Asian pigs and Goettingen Minipigs. The distance between the European and Asian breeds reflects the current scientific consensus that domestication happened independently in Europe and Asia about 9000 year ago (Giuffra et al. 2000). The European breeds appear generally closer to each other, which might be explained, through the different domestication processes in both centers: while the European breeds emerged more or less directly from relatively uniform wild boar strains (Scandura et al. 2008), the Asian domestication history is characterized by complex human driven dispersal of domesticated pigs in the south east Asian archipelagos, sometimes interrupted by feral states, before pigs eventually reached the Asian mainland (Larson et al. 2007). This might explain why the European group clusters closely together in the UPGMA tree with higher resampling support than the Asian group. The tree based on genome wide SNP, clusters on one hand Xiang, Meishan and the South Chinese wild boars and on the other hand Jiangquhai and the North Chinese wild boars together, interrupted by the Mini-LEWE. This is in contradiction to Ai et al. (2015) where Meishan clustered together with the North Chinese wild boars and could also support the low resampling probabilities found among the Asian breeds. The Mini-LEWE, a composite miniature breed, developed by crossing Vietnamese potbellied pigs, Saddlebacks and German Landrace, is in our study represented by a DNA pool of 10 females and a virtual pool made up from two sequenced individuals. Although it appears, that individual sequences are not fully comparable to pools, since we found that mixing of individual and pool sequences leads to clustering of the respective sample types (Results not shown). Still, the virtual and the DNA Mini-LEWE pools are clearly identified as one breed. Therefore the virtual pooling seems to be a suitable measure to make different types of data comparable. In the case of the GMP, both types were mixed and each analysis, PCA and the

UPGMA tree shows, that it is easily discriminable from all other breeds. The phylogenetic tree supports a GMP clade with 100 % resampling support, which is located among the Asian pig breeds. This can be explained by the cross-breeding history in which Vietnamese potbellied pigs, Minnesota Minipigs and German Landrace were involved (Glodek and Oldigs 1981). An earlier study (Gaerke et al. 2014) estimated that about 70 % of the GMP genome are of Asian origin. In the PCA, the first component identifies the difference between the GMP and all others as the main source of variation, accounting for 78 % of the genetic variability. Following the interpretation of Kim et al. (2005), the average F_{ST} between the three groups lying between 0.25 and 0.35 suggests, that still a major part of the total variability can be assigned to differences among individuals. Anyway, albeit using microsatellite data, they encountered similar estimates for F_{ST} values in a set of breeds comparable to this study. Therefore we conclude that the GMP is still a distinct breed that can be easily distinguished from other breeds.

Variation and Differentiation within and between the GMP pools

While it seems particularly easy to distinguish the GMP from other pig breeds, it is more difficult, but relevant from a breeders' point of view, to determine, if genetic isolation of the five breeding units has led to differentiated subpopulations. Applying PCA on the differentiation and distance measures (F_{ST} and D_R) between the ten GMP pools, we were able to see a trend to three subgroups consisting of NR, RE, and a cluster comprising NI and DA, respectively. The presence of a certain level of stratification is expected and has been observed before, i.e. in studies in dogs (Quignon et al. 2007) or sheep (Kijas et al. 2009). In the latter study, several breeds with heterogeneous breeding background split into sub clusters, for example dependent on their origin (American Suffolk vs British Suffolk, F_{ST} ~ 0.058 - 0.064; African vs American Dorpers, F_{ST} = 0.053) or phenotypic differences (Australian Poll Dorset vs American Dorset, F_{ST} = 0.082), while New Zealand and American Texel appeared indistinguishable (F_{ST} = 0.025). Studies comparing clearly distinct pig and cattle breeds, respectively, found F_{ST} values between 0.06 and 0.40 (Ai et al. 2013; McKay et al. 2008). F_{ST} of ~ 0.1 was found between relatively similar breeds, for example Large White and Landrace, while values higher 0.3 indicated major differentiation, such as between Nellore and Holstein cattle or Asian and European pig breeds. These values matched our findings between the European, Asian and GMP groups. Within the GMP, even two randomly composed pools from the same unit had a minimum differentiation of about 0.035. Between the aforementioned clusters F_{ST} was about 0.05 and therefore close to the differentiation

observed between the sheep breeds from separate origins. We explain this by genetic drift and slight differences in the actual breeding management, since the three clusters also belong to the three partners in GMP breeding, even though all follow generally the same breeding goal.

Comparing our results with the F_{ST} levels found in the aforementioned studies implicates that our stocks might be at the edge of splitting into sub-populations, and, when focusing on individual loci, we expected that not all genomic regions between all pairwise combinations of the five units to be similarly differentiated. The F-test (**Table 5.4**) identified about 4 to 14.5 % of the genome to be objected by differentiation which covers the range found in a comparable study by Amaral et al. (2011). We hypothesize that genetic differentiation should be attributed to drift rather than to selection, if it affects neutral loci relatively more than loci with putative harmful consequences on protein translation, such as stop codon gains or deleterious missense mutations. This was supported by an underrepresentation of detrimental variation among highly differentiated loci. The eight loci representing deleterious missense mutations with maximum F_{ST} were located in genes without known sensible relation to traits important in the GMP. Seven of these SNPs are identified when the NR subpopulation is involved in a pairwise comparison, indicating that NR might have drifted apart from the remaining populations relative more than the others. When focusing on the subset of loci where all units are fixed for either allele, it is striking, that RE more often seems to be fixed for the opposite allele compared to the other stocks, as reflected by the negative pairwise correlation of the frequencies with the other units.

While the analysis of single crucial mutations could be misleading due to the complex nature of many traits of interest, integration of systems biology approaches, specifically pathway annotations, might be beneficial (Stranger et al. 2011). The only pathway found differentiated between stocks, DA3 and NR, was ‘Glutamatergic synapse’, regulating the neuronal excitability (Niswender and Conn 2010), which could lead to differences of the neuronal signal transduction. Besides being involved in disorders such as schizophrenia and depression (Sanacora et al. 2008; Meador-Woodruff et al. 2003), constraints in signal transduction are also known to alter the locomotion and behavior of the respective organism (Chiel and Beer 1997; Picetti et al. 1997) and could be explained by hidden selection due to different handling strategies. Anyway, no additional differentiation could be found between one of the two aforementioned stocks and another stock in this pathway. Therefore, differentiation is rather likely to be incidental, than systematic, since handling or phenotype selection on behaviour is the same in DA2 and DA3. When we looked at expected heterozygosity as a measure of

variability, RE and NI exhibit the highest values. It is even more notable, that RE holds more private polymorphisms than all other units together, making it an indispensable resource of genetic variability. We explain this with the consequent implementation of the mating scheme based on the optimum genetic contribution concept (Meuwissen 1997) in RE.

Not only is the preservation of a common genetic identity for all stocks of the GMP important (Bollen and Ellegaard 1997), but also the risk of inbreeding depression and loss of variation due to drift is increased in the artificially reduced subpopulations (Lacy 1987). To counter this in future, two strategies appear feasible: First, the exchange of genetic material, e.g. via artificial insemination, and second, selection of a most diverse set of breeders as basis for future breeding. The first strategy, commonly used by dog breeders, would harbor various risks of spreading diseases and disorders between units. In this scenario, semen from RE, the major reservoir of remaining variability, should be used to inseminate breeders in the other units. The second option of selecting a most diverse set of breeders from the respective unit also has the potential to increase heterozygosity, as can be observed in NI whose founding population was established in that very way. It can be taken as an example of the Bulmer effect (Bulmer 1971) that genetic variation in the relatively large stock of DA3 wasn't lost while selection and assortative mating was conducted, and could be largely recovered when the NI founders were chosen for maximum diversity.

Conclusion

Our study based on assessment of differentiation between the genetically isolated breeding units of the GMP found evidence for stratification, even though it appears less than in other breeds, and the whole GMP breed is still easily discriminable from other pig breeds. Functional annotation revealed, that loci with functional consequences are less differentiated than neutral loci, which implies that the forces differentiating the units appear to be rather drift than selective pressures. The pathway analyses, used to analyze more complex inheritance of traits, could identify just one pathway with possible link to behavior as being differentiated between two stocks.

Albeit animal exchange seems not yet necessary, the RE subpopulation harbors the highest amount of genetic variation and seems to be most similar to the other units in pathways related to human diseases and appears therefore as the potential source for exchange animals.

Competing interests

The authors declare that there are no competing interests.

Author contributions

CR, LFM and HS conceived the study and organized the sampling. CR conducted most analyses and wrote the manuscript. JG assisted in raw data preparation and variant calling, NTH provided the gene-based and pathway tests, ARS designed the F-test-statistic, SW designed and performed DNA extraction and pooling. All authors were involved in interpretation of the results and agreed on the final version of the manuscript.

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Ethics statement

Blood samples were obtained within the course of the regular health monitoring schemes by state approved veterinarians, in accordance with the respective national regulations.

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Supplementary Material

Accessible through: https://figshare.com/s/228890c2f2675409d6cd

Supplementary table 5.1: Genome wide FST (upper triangle) and Reynolds distance (lower triangle)

Supplementary table 5.2: Number of SNPs significant at 5% Bonferroni corrected significance level in F-Test

Supplementary table 5.3: Functional annotation of SNPs oppositely fixed between one unit and a distant pool from another unit

Supplementary table 5.4: Functional annotation of SNPs dependent on the F_{ST} value between two units

CHAPTER 6

General Discussion

General discussion

This thesis aimed to evaluate the potential of next-generation sequencing in the Goettingen Minipig for discovery of the genetic background of miniaturization and to elucidate the effect of population subdivision on the genome. It has taken place during a period of massive progress in the field of sequencing studies on livestock. In retrospect, some decisions naturally would have been taken differently, if the technological progress was foreseeable right from the start. This chapter discusses the important role of the chosen reference genome Sscrofa 10.2 and possible alternatives, as well as the chosen sequencing strategy including sample selection. It addresses what the possible role of highly differentiated functional SNPs might be and how results can be interpreted. The second study focused only on differentiation, so in turn, one section tries to elucidate if our stocks are not only non-differentiated, but if selection is actually targeting the same genes in all stocks. Finally, we present some initial results of a preliminary study on structural variation and its potential impact on the GMP.

The role of the reference genome

Application of next generation sequencing techniques in livestock have provided detailed knowledge about the inheritance and genetic background of relevant traits (Andersson et al. 2012; Imsland et al. 2012; Herrero-Medrano et al. 2014). Nevertheless, few of these studies, even though the term is regularly used, revealed true whole genome sequencing (WGS), which can be understood as the *de-novo* assembly of a complete genome, in the narrow sense. Most of these approaches should rather be considered as what is named whole-genome re-sequencing (WGR, Bentley 2006). In contrast to WGS, WGR relies on a reference genome, which must be assembled beforehand. The samples, mostly sequenced in short reads, can then be mapped against the known reference genome to identify genetic dissimilarities.

The quality of WGR studies is of course dependent on the representation of the regions of interest, which are unknown prior to the analysis, in the chosen reference sequence. An interesting example to illustrate this is coat colour in the swine. Rubin et al. (2012) found that a combination of duplications and a splice mutation underlie the belted and uniformly white coat phenotypes, using the reference sequence of a Duroc pig (Archibald et al. 2010), which itself is of wild-type coat colour. However, if for example, a landrace sow had been chosen as the reference animal, that duplicated structure would not have been identified easily. Therefore, the choice of an appropriate reference genome is of foremost importance for the

success of a re-sequencing study, aiming to elucidate the genetic background of a phenotypic trait, such as growth or body size.

Our analyses relied on the reference genome Sscrofa 10.2 (Groenen et al. 2012) of the aforementioned Duroc sow. Alternatively, the sequence of a highly inbred Wuzhishan pig (WZSP, Fang et al. 2012) was published at the same time. Since the current GMP is a composite breed, expected to have a hybrid genome of Asian-European descent, both genomes would have been a reasonable choice, respecting phylogenetic considerations. Eventually the Duroc was chosen for being the 'official' genome, supported by Ensembl (Yates et al. 2016) and other databases, which provided a convenient infrastructure for analysis beyond variant calling. Unfortunately the GMP reference sequence project, conducted by Glaxo Smith Kline, using an Ellegaard GMP (Vamathevan et al. 2013), was halted before the assembly reached the scaffold or chromosome level, similarly as for the sequence of a Tibetan pig (Li et al. 2013). The build Sscrofa 10.2 was recently replaced by the improved build Sscrofa 11.1 (NCBI 2018). While the build 10.2 was widely used and enabled numerous important discoveries (Groenen 2016), doubts about quality and completeness were inherent. For example, the crucial *IGF2* gene sequence was not contained and Warr et al. (2015) found that about 14 % of the genome was of low quality and 26.6 % of low coverage, which together suggest 33 % of low confidence regions, where variant calling is expected to be compromised. The question arising is, if another reference genome might have been a better choice, or if employing the new reference build would promise drastically new insights? And in the case that no available reference genome would fit the GMP, would it be necessary to assemble a GMP reference genome?

One measure to assess this would be resemblance of the reference genomes against each other. We aligned the existing GMP reference contigs against the two Duroc builds and assessed the proportion of mapped reads (BWA mem alignment, Li and Durbin 2009; Picard sorting, Picard 2009; Samtools flagstat, Li et al. 2009). The same was done, for 200 bp long fragments of the contigs, so called 200mers, in order to mimic the structure of short read data (**Table 6.1**).

Mapping contigs led to a higher number of secondary alignments against both reference genomes, than using the 200mers. Considering only primary alignments of the 200mers, a notable part of the GMP genome, ~4.4 %, could not be mapped against the 10.2, but only to the 11.1 build. Just half a percent could not be mapped at all. Repeating this with our 150 bp paired end sequencing data of a Dalmose DNA pool provided similar results (**Table 6.2**).

This indicates that repeating the experiment with the new reference genome, probably including more samples, could lead to substantially improved results, since we must assume, that about 5 % of the GMP genome are not represented in the 10.2 reference, including important genes, i.e. *IGF2*, and further 30 % of the represented regions are of low confidence. At this point it seems that the new reference genome 11.1 is suitable enough for the GMP and there is no need to develop a GMP reference genome.

Table 6.1: Statistics of mapping the GMP reference against Duroc builds 10.2 and 11.1.

SscrofaMinipig	Contigs		200mers	
	10.2	11.1	10.2	11.1
Raw reads	231.585	231.585	11.908.496	11.908.496
reads total	515.052	320.704	12.309.864	12.072.650
Mapped	507.762	319.889	11.713.065	12.013.643
secondary	283.467	89.119	401.368	164.154
unmapped	7.290	815	596.799	59.007
Mapped	96.85%	99.65%	94.99%	99.50%
mapped [%], incl. Secondary	98.58%	99.75%	95.15%	99.51%
Secondary of mapped reads [%]	55.83%	27.86%	3.43%	1.37%

Table 6.2: Statistics of mapping a Dalmose pool against Duroc reference.

Da_21	10.2	11.1
Raw reads	467.844.016	467.844.016
Reads total	479.698.361	474.464.862
mapped	457.643.218	471.766.607
properly paired	391.281.350	421.321.004
secondary	11.854.345	6.620.846
unmapped	22.055.143	2.698.255
mapped [%]	95.29%	99.42%
mapped [%], incl. Secondary	95.40%	99.43%
Secondary of mapped reads [%]	2.59%	1.40%

Sample selection and sequencing strategy

The costs of sequencing are constantly decreasing (NHGRI 2016) which we experienced in majorly different prices for our two projects, where sequencing was conducted with a time difference of just three years. A mammalian genome at a decent coverage of ~15X would cost about 1,500€ today, which is 10 to 15 times more expensive than array genotyping. This effectively limits the number of samples in a study. A cost effective approach chosen in other studies (Zhu et al. 2012) is to sequence DNA pools instead of individual samples. In pool-seq, equimolar amounts of DNA from individuals are pooled and sequenced as one sample, which makes it impossible to determine the origin of each read or construct long haplotypes from short reads, without further methods, such as barcoding. It has been shown that allele frequencies, estimated from reads counts, are sufficiently reliable (Anand et al. 2016; Zhu et al. 2012). Still, there are some disadvantages: Pool-seq is inherently afflicted with a bias introduced by pooling and sequencing errors (Kofler et al. 2011), which is particularly due to a problem of differentiating between these error types and real rare variants. Rare variants are defined as variants with a minor allele frequency less than 1 % (Anand et al. 2016) to less than 5 % (Kim et al. 2010) and are assumed to explain a major part of genetic variation (Anand et al. 2016). Furthermore, many current statistical approaches to identify rare variants were initially not designed for pool-seq data (Wang et al. 2010b). In addition, F_{ST} estimates from pooled and individual sequences seem to be differently distributed (Bersaglieri et al. 2004; Akey et al. 2002), a finding we also noticed in **chapter 5**. A possible explanation is a high number of falsely positive detected differentiations, when either coverage or sample size are too low. Lynch et al. (2014) estimated that at minimum of both 100 samples and 100X coverage are necessary to reliably asses differentiation and reduce the number of false positives. On the other hand, sequencing much deeper is not seen as an improvement. Approximately 1X coverage per animal per sample in a pool should be optimal in terms of cost-efficiency (Lynch et al. 2014). While we sequenced our pools in **chapters 4** and **5** to sufficient depth according to that study, our sample sizes were far below those suggested, implying that a significant proportion of the differentiated variants observed may be false positives. Another strategy that we took in **chapters 2** and **3**, is to estimate allele frequencies from genotypes based on individual sequencing. Kim et al. (2011) outlined that this should be done with caution when individual samples were sequenced at low to medium coverage (< 15X), since again, rare allele estimates are expected to be biased. If it was necessary, genotypes should not be filtered on genotype confidence, which we actually performed, albeit

in a relatively lenient manner. Despite all these possible disadvantages, pool sequencing has frequently proven to be especially useful to enlighten the background of interesting traits, albeit often violating the aforementioned rules (Carneiro et al. 2014; Rubin et al. 2010; Lamichhaney et al. 2012).

The major strategical advantage of preferring pool-seq over individual sequencing is the larger number of samples to be included at the same cost, which facilitates incorporation of multiple breeds or multiple strains of the same breed. This in turn is expected to improve the power of differentiation studies by eradicating artefacts based on stratification (Schlötterer 2002; Zhu et al. 2012). We tried to incorporate this by including a second miniature breed, the MiniLEWE in **chapters 2** and **3**, and by sequencing two pools per stock as described in **chapters 4** and **5**. With a focus on body size, breeds to be involved in future could be Bama pigs from China (Liu et al. 2008) or the pygmy hog Sus salvanius/ Porcula salvania (Funk et al. 2007). One point that should be mentioned is, that all these analyses including multiple miniature breeds, are based on the hypothesis that their miniaturization has a common genetic background, which is debatable with respect to the situation in humans (Merimee et al. 1989; Klingseisen and Jackson 2011; Mayer et al. 2001).

Figure 6.1 shows expected heterozygosity, estimated from the GMP pools from the second study (**chapters 4** and **5**) and the MiniLEWE and GMP samples from the first study (**chapters 2** and **3**). Chromosome 5 carries a region we identified as a major selective sweep between 40 to 46 Mb in the first study, which could serve as an example for different results due to different experimental setting as they were mentioned before. Revisiting the same region in the pool data, the strong signature is not observed. Possible explanations could be different variant calling algorithms, different filtering strategies, the aforementioned differences between pool and individual sequencing or simply a sampling error due to a low number of GMP samples. However, in both types of data, this region shows irregular behavior, elevated heterozygosity in the pools and diminished heterozygosity in the old samples, which also incorporated a MiniLEWE pool of ten sows. Both datasets show concordance in the remaining genome, but we feel that this issue cannot be resolved based on the current analyses and requires further research.

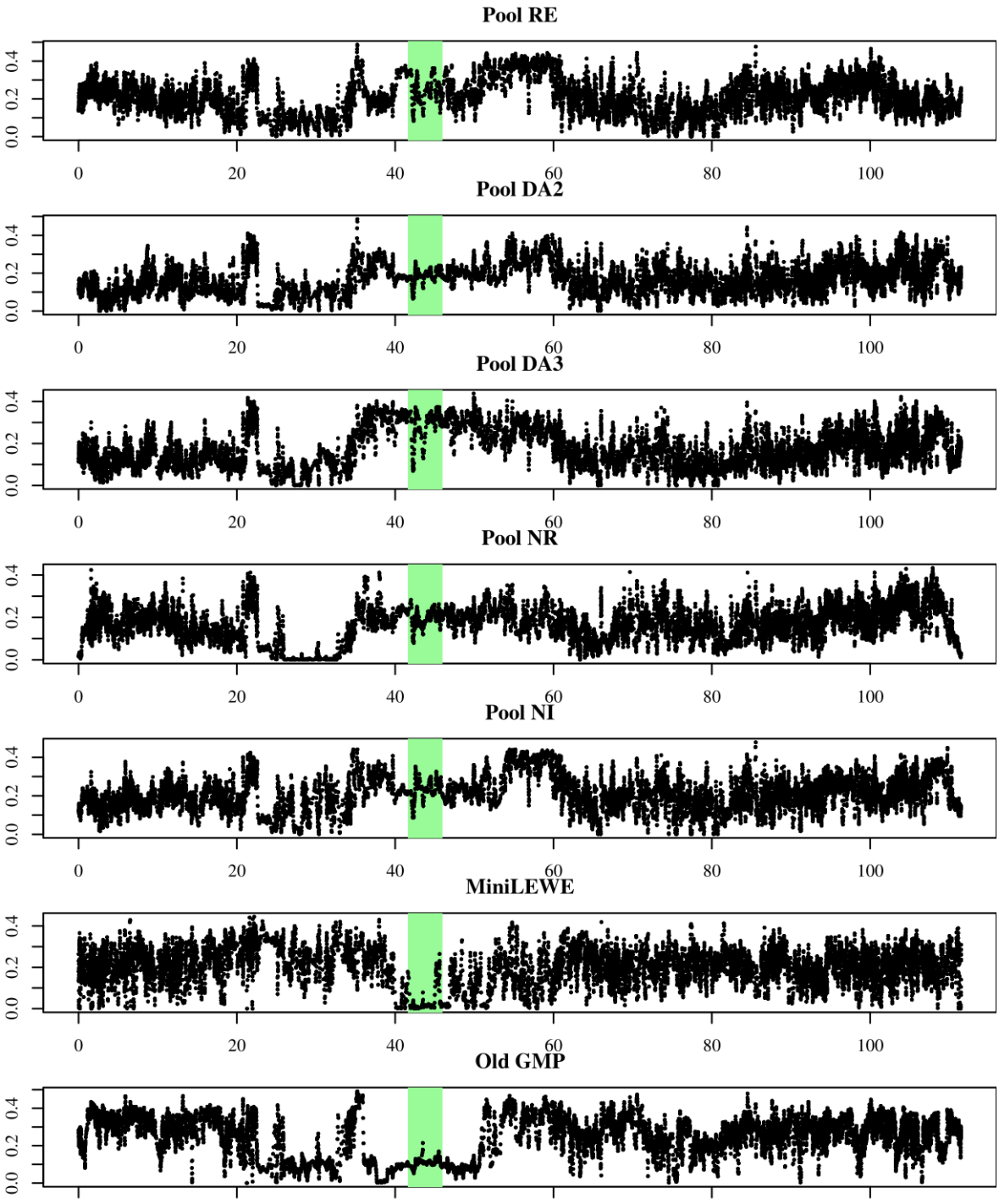

Figure 6.1: Expected heterozygosity, averaged in 250 SNP windows along chromosome 5. Respective sweep region highlighted in green.

Differentiation

Single nucleotide mutations can have a tremendous effect on the functionality of proteins and subsequent phenotypes (Amorim et al. 2017). Studies aiming at identification of nonsense alleles underlying genetic diseases were fairly successful in associating, for example, stop codons with dementia (Vidal et al. 1999), legionnaires disease (Hawn et al. 2003) or stickler syndrome (Ahmad et al. 1991). Conte et al. (2017) found that deleterious mutations were effectively under selection in spruce and therefore reduced in allele frequency compared to non-deleterious mutations. F_{ST} has been successfully used to efficiently map regions of divergent selection in the herring (Lamichhaney et al. 2012). Therefore, applying combined F_{ST} analysis and functional annotation using WGS data is a promising approach to identify loci undergoing divergent selection, or having been fixed by it. The most representative example of such a locus might be gait pattern in horses (Andersson et al. 2012), where a premature stop codon mutation in *DMRT3* determines if a horse has four or five, instead of three gaits. Breeds able to do pacing or trotting, like the Icelandic horse, were found to carry the mutation at high allele frequencies or even show fixation for the mutation.

We used similar approaches in our studies aiming to identify the background of growth and evaluating the effects of differentiation in the GMP stocks. In the first study, we identified autosomal loci with high F_{ST} values, which will include oppositely fixed loci, and annotated them using the Ensembl variant effect predictor (McLaren et al. 2016). As shown in **Figure 6.2**, only 1,331 SNPs at $F_{ST} > 0.95$ and 804 SNPs thereof at $F_{ST} = 1$ were detected, respectively. Few were annotated to multiple functional classes.

The majority of these loci are located in intergenic regions, where mechanisms of possible functional constraint remain poorly understood. No missense mutations were identified. The coding sequence variants were located in *DNAJC28*, involved in Golgi vesicle transports (Yates et al. 2016), *ITGB2* underlying leukocyte adhesion deficiency in cattle and dogs (Daetwyler et al. 2014; Kijas et al. 1999), *CHD6*, involved in gene regulation (Lathrop et al. 2010) and a novel gene. No obvious relations to GMP phenotypes were evident. Similarly, a study on domestication of rabbits found that few loci go towards fixation and none of them were located in coding regions (Carneiro et al. 2014). Rafati et al. (2016) located the few high F_{ST}-SNPs they identified in a case-control study on skeletal atavism in horses, only on unassigned scaffolds.

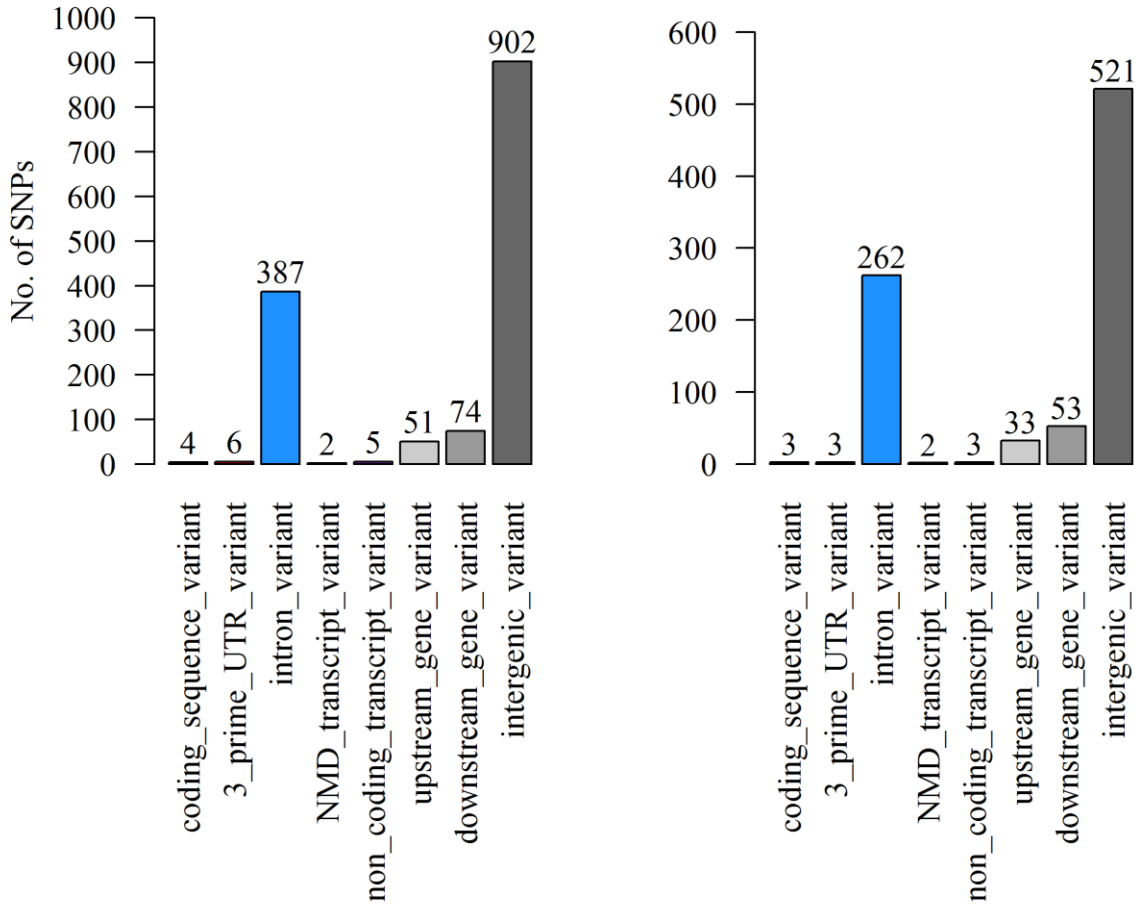

Figure 6.2: Functional annotation of high F_{ST} values. Left: F_{ST} > 0.95; Right: F_{ST} = 1.

This might imply, single highly differentiated SNPs may have limited relevance in the genetics of complex traits, such as growth or fertility, which we focused on. Conversely, using highly differentiated missense mutations as indicators for functional divergence of the GMP stocks, as we did in **chapter 4** and **5**, might fall short and critically underestimate real divergence. Christe et al. (2017) showed that despite generally high differentiation, no fixed polymorphisms appeared in a certain region. Additionally, if deleterious mutations were beneficial for a desired phenotype, these could interact through genetic complementation without single loci necessarily being fixed (Conte et al. 2017). Sohail et al. (2017) also found that deleterious mutations seem to function synergistically rather than independently, which would imply that multiple deleterious mutations may have a stronger effect than a single locus. This leads to negative linkage disequilibrium between deleterious mutations and would support the hypothesis of complementation. In any case, it appears that, apart from qualitative traits and few examples, highly differentiated deleterious mutations do not play a major role in complex traits in livestock.

Not differentiated, but selected for the right aim?

Chapters 4 and **5** focused on differentiation between the five GMP production stocks, due to the interest surrounding if the stocks had diverged in traits important for the functionality of the GMP as a model animal. Conversely, reduced differentiation in genomic regions underlying the goal traits, fertility and growth could be interpreted as evidence that the current breeding scheme affects the same genes in all stocks similarly. As the study on growth demonstrated, the genetic background of growth is polygenic rather than oligogenic, and fertility is already a multifactorial (probably highly polygenic) and not easy to define trait. An important measure of fertility in pigs is litter size, which is influenced by various parameters (Clark et al. 1988); among others male fertility (Lawlor and Lynch 2007), prenatal mortality (Spötter and Distl 2006) and ovulation rate (Rothschild et al. 1997). It is known from sheep that single genes with major effect exist, e.g. the Booroola gene (Fogarty 2009; Davis 2004), and the estrogen receptor *ESR* is associated with a major QTL for litter size in pigs (Rothschild et al. 1997), although this seems to be highly variable between different populations (Drogemuller et al. 2001; Wu et al. 2006). Otherwise, most fertility traits in common populations presumably have a polygenic background (Ferlin et al. 2007; Chen et al. 2001; Davis 2004). In such case, pathway analysis is expected to outperform approaches focusing on single loci (Torkamani et al. 2008). Using the KEGG based pathway analyses described in **chapter 5**, but focusing on pathways that were especially undifferentiated, resulted in 83 out of the 316 total pathways identified. The majority of these pathways belong to the functional classes 'Metabolism', 'Organismal Systems' and 'Human Diseases'. The stocks with the highest number of undifferentiated pathways were NI in 'Metabolism' and 'Environmental Information Processing' (EIP), NR in 'Organismal Systems', 'Human Diseases' and 'Cellular Processes' (together with NI) and DA2 in 'Genetic Information Processing' (GIP). Lowest frequencies were seen for DA3 in 'Metabolism', for RE in 'Organismal Systems', 'Human Diseases' and 'Cellular Processes', for DA3 in 'GIP' and 'EIP' (**Figure 6.3**). Pathways were rarely ever undifferentiated between all units in all contrasts (**Figure 6.4**). The only example for this is "Olfactory transduction", but mostly, a unit was only significantly indifferent against one or two other units. Higher numbers were, for example, seen in 'Steroid hormone biosynthesis' (NR vs RE and DA3), 'Retinol metabolism' (DA2 vs RE, DA3 and NR), or 'Fluid shear stress and artherosclerosis' (NI vs DA2, DA3 and NR).

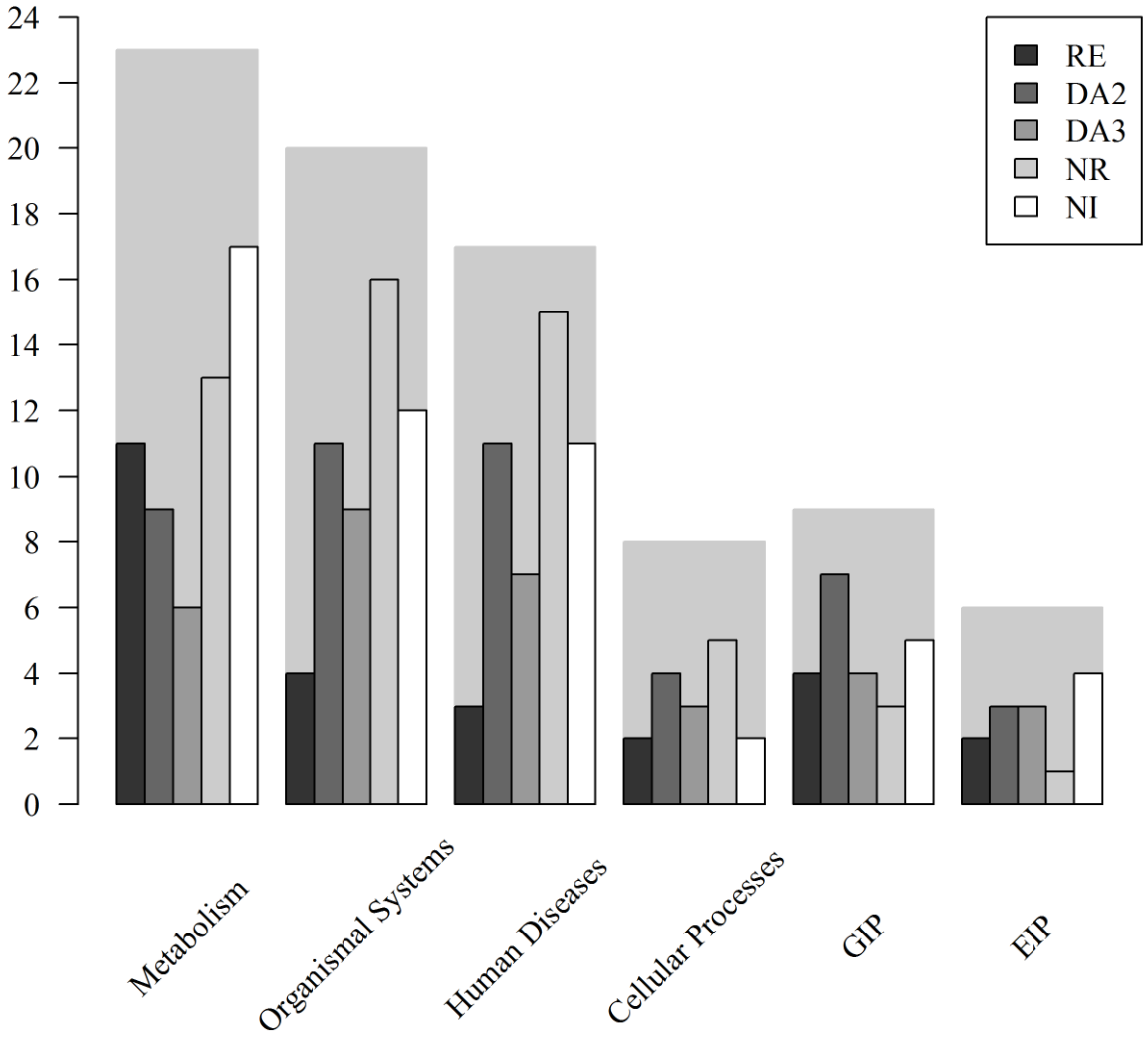

Figure 6.3: Number of pairwise significantly undifferentiated pathways by stock and functional category of pathway. Grey shading indicates number of pathways per class showing any significant similarity. GIP/ EIP = Genetic/ Environmental information processing.

'Olfactory transduction', the only pathway undifferentiated between all stocks has been found to be associated with highly conserved genomic regions, putatively under balancing selection, in an earlier study on domestic and wild pigs (Amaral et al. 2011). Olfactory perception as one of the basic sensory functions is important in many species and similarly organized (Ache and Young 2005). The underlying gene family is one of the largest, with a notable proportion of pseudogenes (Ache and Young 2005; Gilad et al. 2003), and genes exhibiting higher heterozygosity than expected (Alonso et al. 2008). 'Steroid hormone biosynthesis' is directly linked to reproductive performance (Penning et al. 2000; Dohle et al. 2003) and has been found to be differentiated between Asian and European pigs (Leno-Colorado et al. 2017). This might imply that selection for reproduction in the GMP might have favoured alleles from

one of the two origins, likely before the population was divided into multiple breeding units. ‘Retinol metabolism’, important for visual function (Blomhoff and Blomhoff 2006), ‘Fluid shear stress and atherosclerosis’ and ‘Chemical carcinogenesis’ are important in respective medical studies and missing differentiation between the units might have positive implications for similar testing behavior of the animal model. In conclusion, general similarities were rare, but it seems that at least selection for reproductive performance might have left particular traces in the genome and the relatively high number of metabolic pathways could be evidence for selection on growth.

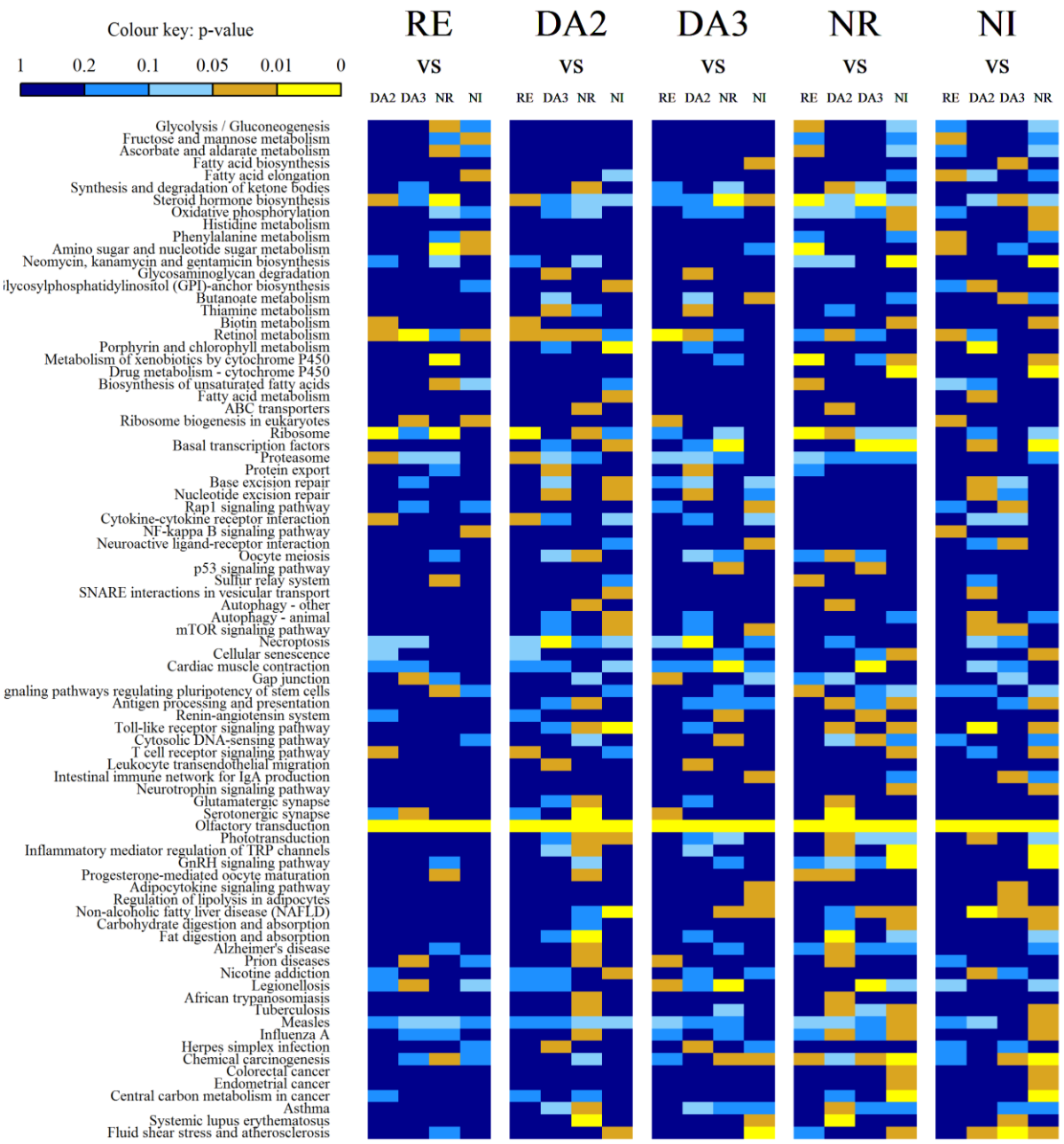

Figure 6.4: P-values of Kolmogorov-Smirnov based pathway test. Low values indicate no differentiation.

Structural variation

All analyses presented in this thesis ignore other types of variation than biallelic SNPs, such as short InDels and all forms of larger structural variation, as well as multi-allelic SNPs. Nonetheless, it is undisputed that structural variation plays a significant role in genetics, while there are different opinions regarding the amount of genetic variation they explain: According to Tattini et al. (2015) they account for about 1 % of genetic variation, while Stankiewicz and Lupski (2010) argue they might have higher impact than biallelic SNPs.

Famous examples of livestock phenotypes related to structural variation are muffs and beard in chicken, based on multiple duplications (Guo et al. 2016), an inversion underlying comb morphology (Imsland et al. 2012) and colour patterns in swine, also regulated by duplications (Rubin et al. 2012). Structural variation (SV), such as inversion or deletions is also likely to have deleterious effect on gene function, as demonstrated by Imsland et al. (2012), where disruption of CCDC108 located at an inversional breakpoint impairs sperm motility in cocks.

We used DELLY (Rausch et al. 2012) to call autosomal inversions, deletions and duplications from the individually sequenced GMP samples (**Chapters 2** and **3**) and restricted calls to a minimum length of 500 bp and maximum length of 25 Mb. We annotated genes (Yates et al. 2016) in all deletions (DEL) and inversions (INV), where at least 80 % of the samples were heterozygous or homozygous for the SV compared to the reference genome and all duplications (DUP) which were exhibited by all samples. These were in total 2,929 DEL (median length 1,257 bp), 5,065 DUP (median length 43 kb) and 2,149 INV (median length 61 kb). **Figure 6.5** shows the length of the genome covered by SVs, in total about 11.6 % of the Duroc reference length. As a proof of principle for the method, the duplicated structures at the *KIT* locus were detected as they would have been expected in a uniformly white coated pig (Rubin et al. 2012). Additionally SVs were co-located with several candidate genes for growth (**Figure 6.6**), such as *TGFβ2* and *TGFβRAP1*, and a large inversion spans the position of the growth hormone receptor *GHR*, a gene known to maintain regular size in pigs and leading to miniaturization when impaired (Cyranoski 2015). These first results indicate that structural variation is inherent in the GMP and is likely to play an important role in its genome, but further research is needed to validate these initial findings. Additional information can be found in Reimer and Simianer (2016).

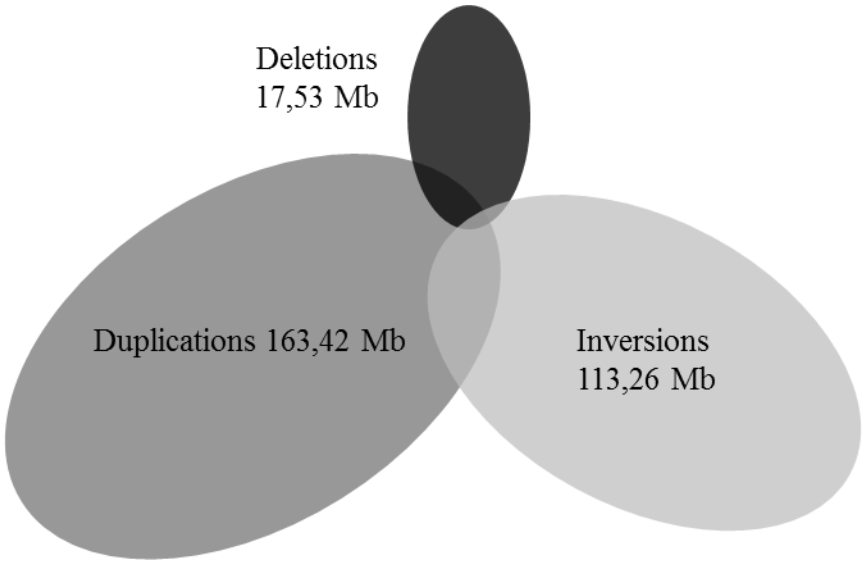

Figure 6.5: Total length of deletions, inversions and duplications in GMP samples.

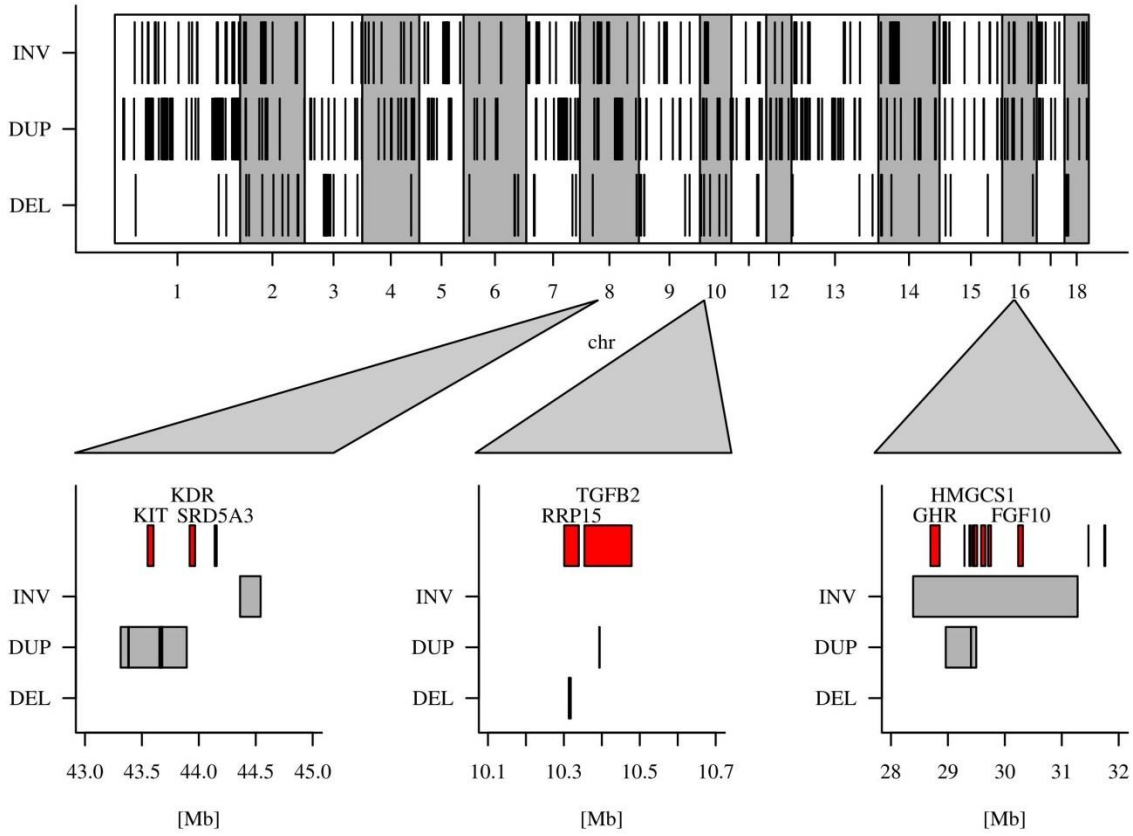

Figure 6.6: Genome wide distribution of duplications (DUP), inversions (INV) and deletions (DEL) and three selected regions around the *KIT* locus, *TGFB2* and *GHR*.

General conclusion

This study demonstrates the capability of NGS to detect signatures of selection from small numbers of miniature pigs and to assess population structure among sub-populations of the same breed. It was conducted during a period of major advancements in genome research on livestock, using whole genome re-sequencing. Here we would like to emphasize some of the findings which could be a help to future projects. Despite ongoing decreases in sequencing costs, NGS is still a relatively expensive technique, limiting sample sizes. If the aim of a study is to analyze the genetic background of a trait by comparison between two groups expressing the trait of interesting differently, using pool-sequencing could be beneficial through inclusion of more samples from different strains or breeds. In any case, multiple reference genomes are available, the best suited should be identified beforehand, to ensure high mapping rates of the re-sequenced genomes. We found that, even though deleterious mutations are an obvious candidate to underlie traits of interest or to explain differences between different sub-populations, the prevalence of highly differentiated deleterious mutations seems to be low and they might rarely be of high relevance for our analyzed traits. Alternatively pathway analyses seem to be a particularly powerful tool to integrate biological information into quantitative analysis and appear well suited to characterize the genetics behind quantitative traits such as fertility. Also, the importance of structural variation seems to be widely underestimated and we suggest, its incorporation in similar future studies should be strongly considered.

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APPENDIX

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8/2011 Synbreed Summer School, Herrsching, Bavaria "Next generation sequence analysis: Practice and departure to new frontiers"

10/2012 ESF Summer School, Pag, Croatia“Livestock: “Conservation Genomics: Data, Tools and Trends”, LivConGen2012 GRANT financed by the European Science Foundation (ESF)

10/2014 Synbreed Summer School, Herrsching, Bavaria “From SNPs to gene netwoks”

8/2015 EAAP/CUP “Workshop on Writing and Presenting Scientific Papers”, Warsaw, Poland

2/2016 Course of the Society for Animal Sciences (GfT) „Statistische Methoden in Quantitativer Genetik und Tierzüchtung“ (Statistical Methods in Quantitative Genetics and Animal Breeding), Schwarzenau, Germany

Teaching

10/ 2012 Excercise on linkage disequilibrium and effective population size, ESF Summer School “Livestock: ‘Conservation Genomics: Data, Tools and Trends’”, Pag, Croatia

WS 2012/13 Lecture „Kopplungsungleichgewicht“ (Linkage Disequilibrium), Angewandte Methoden der Tierzucht, Master courses Agricultural sciences, GAU Göttingen

SS 2015 Lecture „Selektionstheorie“ (Selection theory), Quantitativ-genetische Methoden der Tierzucht, Masters course Agricultural sciences, GAU Göttingen

SS 2015 Weekly excercises, Quantitativ-genetische Methoden der Tierzucht, Masters course Agricultural sciences, GAU Göttingen

SS 2016 Weekly excercises, Quantitativ-genetische Methoden der Tierzucht, Masters course Agricultural sciences, GAU Göttingen

SS 2017 Weekly excercises, Quantitativ-genetische Methoden der Tierzucht, Masters course Agricultural sciences, GAU Göttingen

WS 2017/18 Lectures and excercises, Quantitative Genetics and Population Genetics, Masters course International Plant and Animal Breeding, GAU Göttingen

Scholarships and grants

10/2012 LivConGen2012 GRANT of the European Science Foundation (ESF) for ESF Summer School auf Pag, Kroatien

4/2013	ESF Exchange Grant 'Advances in Farm Animal Genomic Resources' for first research stay in Uppsala
11/2013	DAAD U4 Netzwerk travel grant for second stay in Uppsala
8/2014	Travel grant Göttingen international for participation in 10th WCGALP in Vancouver, Canada
8/2015	EAAP Conference scholarship 2015 for the society's annual meeting in Warsaw and the associated course „Writing and presenting papers“

Publications

Refereed publications

Reimer, C., Rubin, C.-J., Weigend, S., Waldmann, K.-H., Distl, O., Simianer, H. (2014) The Minipig Genome Harbors Regions of Selection for Growth. Proceedings, 10th World Congress of Genetics Applied to Livestock Production, Vancouver, Kanada

Gholami, M., **Reimer, C.**, Erbe, M., Preisinger, R., Weigend, A., Weigend, S., Servin, B., Simianer, H. (2015) Genome Scan for Selection in Structured Layer Chicken Populations Exploiting Linkage Disequilibrium Information. *PLOS One*, DOI: 10.1371/journal.pone.0130497.

Ni, G., Strom, T.M., Pausch, H., **Reimer, C.**, Preisinger, R., Simianer, H., Erbe, M. (2015) Comparison among three variant callers and assessment of the accuracy of imputation from SNP array data to whole-genome sequence level in chicken. *BMC Genom*., **16**:824, doi:10.1186/s12864-015-2059-2.

Ha, N.T. Drögemüller, C., **Reimer, C.**, Schitz-Hsu, F., Bruckmaier, R.M., Simianer, H., Gross, J.J. (2017) Liver transcriptome analysis reveals important factors involved in the metabolic adaptation of the transition cow. *J Dairy Sci*. doi: **10**.3168/jds.2016-12454.

Malomane, D.K., **Reimer, C.**, Weigend, S., Weigend, A., Simianer, H. (2018) Efficiency of different strategies to mitigate ascertainment bias when using SNP panels in diversity studies. *BMC Genomics*. **19**: 22

Reimer, C., Ha, N.-T., Sharifi, A.R, Weigend, S., Geibel, J., H. Simianer, H. (2018) Analyses of the breed integrity of the Goettingen Minipig using pool-sequencing. Proceedings, 11th World Congress of Genetics Applied to Livestock Production, Auckland, New Zealand.

Geibel, J., Weigend, S., Weigend, A., **Reimer, C.**, Pook, T., Simianer, H. (2018) Array Design and SNP Ascertainment Bias. Proceedings, 11th World Congress of Genetics Applied to Livestock Production, Auckland, New Zealand.

Malomane, D.K., **Reimer, C.**, Weigend, S., Weigend, A., Simianer, H (2018). The contribution of genomic regions to genetic variation in global chicken populations. Proceedings, 11th World Congress of Genetics Applied to Livestock Production, Auckland, New Zealand.

Martini, J.W.R., Schrauf, M.F., Garcia-Baccino, C.A., Pimentel, E.C.G., Munilla, S., Rogberg-Munoz, A., Cantet, R.J.C., **Reimer, C.**, Gao, N., Wimmer, V., Simianer, H.

(accepted 2018) The effect of the H^-1 scaling factors Tau and Omega on the structure of H in the single-step procedure. *Genetics Selection Evolution*.

Cardoso, D.F., Albuquerque, L.G., **Reimer, C.**, Qanbari, S., Erbe, M., Vieira do Nascimento, A., Venturini, G.C., Becker Scalez, D.C., Baldi, F., Camargo, M.F., Mercadente, M.E.Z., Goncalves Cyrillo, J.N., Simianer, H., Tonhati, H. (accepted 2018) Genome-wide scan reveals population stratification and footprints of recent selection in Nelore cattle. *Genetics Selection Evolution*.

Reimer, C., Rubin, C.-J., Sharifi, A.R., Ha, N.-T., Weigend, S., Waldmann, K.-H., Distl, O., Pant, S.D., Fredholm, M., Schlather, M., Simianer, H. (submitted to BMC Genomics) Analysis of porcine body size variation using re-sequencing data of miniature and large pigs.

Reimer, C., Ha, N.-T., Sharifi, A.R., Weigend, S., Geibel, J., Mikkelsen, L..F., Weigend, S., Simianer, H. (in preparation) Assessing breed integrity of the Goettingen Minipig.

Theatre presentations

Reimer, C., Rubin, C.-J., Simianer, H. (2013) Die Diversifikation von Minischweinen von anderen Schweinerassen auf der Basis von genomweiten Sequenzdaten. Annual meeting of the DGfZ, Göttingen, Germany.

Gholami, M., **Reimer, C.**, Erbe, M., Preisinger, R., Weigend, A., Weigend, S., Servin, B., Simianer, H. (2014) Genome scan for selection signatures in layer chicken populations. Annual meeting of the DGfZ, Dummerstorf, Germany.

Reimer, C., Kramer, M., Erbe, M., Bapst, B., Bieber, A., Simianer, H. (2014) Novel Functional Traits in Low Input Dairy Cattle: Genetic Characterisation and the Potential of Genomic Selection. LowinputBreeds final meeting, Newcastle University, Newcastle, United Kingdom.

Reimer, C., Rubin, C.-J., Weigend, S., Waldmann, K.-H., Distl, O., Simianer, H. (2014) The Minipig Genome Harbors Regions of Selection for Growth. 10^{th} World Congress of Genetics Applied to Livestock Production, Vancouver, Kanada.

Reimer, C., Rubin, C.-J., Simianer, H. (2014) Warum ist das Minischwein so klein? Seminar für Nutztierwissenschaften, Göttingen, Germany.

Reimer, C., Schlather, M., Distl, O., Simianer, H. (2014) Jeder Signatur ihr Beobachtungsfenster: Flexible Dimensionierung von Beobachtungsfenstern bei der

Suche nach Selektionssignaturen im Minischweinegenom. Annual meeting of the DGfZ, Dummerstorf, Germany.

Reimer, C., Yin, T., Bapst, B., Simianer, H., König, S. (2014) Genetic analysis of Brown Swiss cows from low input farms in Switzerland. LowinputBreeds final meeting, Newcastle University, Newcastle, United Kingdom.

Ni, G., Strom, T.M., Pausch, H., **Reimer, C.**, Preisinger, R., Simianer, H., Erbe, M. (2015) Accuracy of imputation from SNP array data to sequence level in chicken. Annual meeting of the DGfZ, Berlin, Germany.

Cardoso, D.F., Venturini, G.C., Mercadante, M.E.Z., Cyrillo, J.N.S.G., Erbe, M., **Reimer, C.**, Simianer, H. (2015) Use of Wright´s fixation index (Fst) to detect potential selective sweeps in Nelore Cattle. EAAP/ EVT annual meeting, Warsaw, Poland.

Reimer, C., Rubin, C.-J., Weigend, S., Waldmann, K.H., Distl, O., Simianer, H. (2015) Analysis of resequencing data suggests that the minipig carries complex disease alleles. EAAP/ EVT annual meeting, Warsaw, Poland.

Reimer, C., Sharifi, A.R., Rubin, C.-J., Weigend, S., Waldmann, K.-H., Distl, O., Pant, S.D., Fredholm, M., Simianer, H. (2015) Ein außergewöhnlicher Haplotyp auf Chromosom X des Minischweines und sein Effekt auf die Körpergröße. Annual meeting of the DGfZ, Berlin, Germany.

Reimer, C., Sharifi, A.R., Rubin, C.-J., Weigend, S., Waldmann, K.H., Distl, O., Pant, S.D., Fredholm, M., Simianer, H. (2015) Sequenzbasierte Analyse der Körpergröße im Vergleich von Mini- und Großschweinen. Genetics-statistical committee of the DGfZ e.V., Bad Sassendorf, Germany.

Malomane, D.K., **Reimer, C.**, Weigend, S., Sharifi, A.R., Simianer, H. (2016) Comparisons of allele frequencies estimated from whole genome re-sequencing data and high-density genotyping array in chicken. Annual meeting of the DGfZ, Hanover, Germany.

Reimer, C., Simianer, H. (2016) Untersuchung großer struktureller Variationen im Genom des Göttinger Miniaturschweins. Annual meeting of the DGfZ, Hanover, Germany.

Reimer, C., Sharifi, A.R., Rubin, C.J., Weigend, S., Waldmann, K.H., Distl, O., Pant, S.D., Fredholm, M., Simianer, H. (2016) A large X-chromosomal haplotype is associated with small body size of minipigs. EAAP/ EVT annual meeting, Belfast, United Kingdom.

Sharifi, A.R., **Reimer, C.**, Ha, N.T., Erbe, M., Cavero, D., Preisinger, R., Simianer, H. (2016) Genetic Analysis of Feather Pecking and Mortality in Laying Hens. EAAP/ EVT annual meeting, Belfast, United Kingdom.

Geibel, J., Weigend, S., Weigend, A., **Reimer, C.**, Pook, T., Simianer, H. (2017) Array Design und SNP Ascertainment Bias. Annual meeting of the DGfZ, Stuttgart, Germany.

Malomane, D.K., **Reimer, C.**, Weigend, S., Weigend, A., Sharifi, A.R., Simianer, H. (2017) The effectiveness of different filtering strategies to reduce the effects of ascertainment bias when using SNP panels in a chicken diversity study. WPSA 2017, Saint-Malo, France.

Reimer, C., Geibel, J., Weigend, S., Simianer, H. (2017) Analyse der Rassenintegrität des Göttinger Miniaturschweins anhand gepoolter Sequenzdaten. Annual meeting of the DGfZ, Stuttgart, Germany.

Reimer, C., Ha, N.-T., Sharifi, A.R, Weigend, S., Geibel, J., H. Simianer, H. (2018) Analyses of the breed integrity of the Goettingen Minipig using pool-sequencing. 11th World Congress of Genetics Applied to Livestock Production, Auckland, New Zealand.

Posters

Malomane, D.K., **Reimer, C.**, Weigend, S., Weigend, A., Sharifi, A.R., Simianer, H. (2017) The effectiveness of different filtering strategies to reduce the effects of ascertainment bias when using SNP panels in a chicken diversity study. WPSA 2017, Saint-Malo, Frankreich.

Geibel, J., Weigend, S., Weigend, A., **Reimer, C.**, Pook, T., Simianer, H. (2018) Array Design and SNP Ascertainment Bias. 11th World Congress of Genetics Applied to Livestock Production, Auckland, New Zealand.

Malomane, D.K., **Reimer, C.**, Weigend, S., Weigend, A., Simianer, H (2018). The contribution of genomic regions to genetic variation in global chicken populations. 11th World Congress of Genetics Applied to Livestock Production, Auckland, New Zealand.

Anlage 3: E r k l ä r u n g e n

1. Hiermit erkläre ich, dass diese Arbeit weder in gleicher noch in ähnlicher Form bereits anderen Prüfungsbehörden vorgelegen hat.

   Weiter erkläre ich, dass ich mich an keiner anderen Hochschule um einen Doktorgrad beworben habe.

   Göttingen, den .....

   .....

   (Unterschrift)

2. Hiermit erkläre ich eidesstattlich, dass diese Dissertation selbständig und ohne unerlaubte Hilfe angefertigt wurde.

   Göttingen, den .....

   .....

   (Unterschrift)