

# Computational challenges in genome wide association studies: data processing, variant annotation and epistasis

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## Preface & Contribution of Authors

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## **Abstract**

Abundant genome sequence information from large cohorts of individuals can now be routinely obtained and this information is poised to ease the identification of genetic variations linked to complex disease. In this work, I investigate the computational and statistical challenges involved in the analysis of large genomic datasets and I tackle three different aspects of the analysis, each of them having very different characteristics. First, in order to analyse large amounts of data from genomic studies we design a programming language, BigDataScript, that simplifies the creation of robust and scalable data analysis pipelines. Second, we create genomic variant annotation and prioritization methods (SnpEff and SnpSift) that help to calculate putative genetic effects and estimate the genetic impact of variants. Finally, we address the problem of finding associations between interacting genetic loci and disease by proposing a methodology that combines population-level genetic information with evolutionary information in order to increase the statistical power in epistatic genome wide association studies

## Abrégé

Il est aujourd’hui possible d’obtenir la séquence du génome de grandes cohortes d’individus, et cette information est permet de faciliter l’identification de variations génétiques liées à des maladies complexes. Dans ma thèse, j’étudie les défis informatiques et statistiques liés à l’analyse de grands ensembles de données génomiques. J’aborde trois aspects de l’analyse. Premièrement, afin d’analyser de grandes quantités de données provenant d’études génomiques nous concevons un langage de programmation, BigDataScript, qui simplifie la création de pipelines d’analyse de données robustes et évolutives. Deuxièmement, nous créons deux méthodes d’annotation et de classification de variantes génomiques (SnpEff et SnpSift) qui aident à prédire leur l’effet possible. Enfin, nous abordons le problème de l’identification de liens entre les maladies génétiques et les variantes qui les causent en proposant une méthodologie qui combine l’information génétique au niveau d’une la population avec informations évolutive afin d’augmenter la puissance statistique des études d’association considérant les interactions épistatiques.

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# Chapter 1

## Introduction

### 1.1 Introduction

How does one’s DNA influence their risk of getting a disease? Contrary to popular belief, your future health is not “hard wired” in your DNA. Only in a few diseases, referred as “Mendelian diseases”, are there well known, almost certain, links between genetic mutations and disease susceptibility. For the majority of what are known as “complex diseases”, such as cancer or diabetes, genomic predisposition is subtle and, so far, not fully understood and involves interaction among several genes as well as between genes and the external environment.

With the rapid decrease in the cost of DNA sequencing, the complete genome sequence of large cohorts of individuals can now be routinely obtained. This wealth of sequencing information is expected to ease the identification of genetic variations linked to complex traits. In this work, I investigate the analysis of genomic data in relation to complex diseases, which offers a number of important computational and statistical challenges. We tackle several steps necessary for the analysis of sequencing data and the identification of links to disease. Each step, which corresponds to a chapter in my thesis, is characterized by very different problems that need to be addressed.

- i) The first step is to analyse large amounts of information generated by DNA sequencers to obtain a set of “genomic variants” present in each individual. To address these big data processing problems, Chapter 2 shows how we designed a programming language (BigDataScript [41]), that simplifies the creation robust, scalable data pipelines.
- ii) Once genomic variants are identified, we need to prioritize and filter them to discern which variants should be considered “important” and which ones are likely to be less relevant. We created the SnpEff & SnpSift [39, 40] packages that, using optimized algorithms, solve several annotation problems: a) standardizing the annotation process, b) calculating putative genetic effects, c) estimating genetic impact, d) adding several sources of genetic information, and e) facilitating variant filtering.
- iii) Finally, we address the problem of finding associations between interacting genetic loci and disease. One of the main problems in GWAS, known as “missing heritability”, is that most of the phenotypic variance attributed to genetic causes remains unexplained. Since interacting genetic loci (epistasis) have been pointed out as one of the possible causes of missing heritability, finding links between such interactions and disease has great significance in the field. We propose a methodology to increase the statistical power of this type of approaches by combining population-level genetic information with evolutionary information.

In a nutshell, this thesis addresses computational, analytical, algorithmic and methodological problems of transforming raw sequencing data into biological insight in the aetiology of complex disease. In the rest of this introduction we give the background that provides motivation for our research.

## 1.2 Genomes and genetic variants

DNA is composed of four basic building blocks, called “bases” or “nucleotides” [6]. These four nucleotides, usually abbreviated  $\{A, C, G, T\}$ , are Adenine, Cytosine, Guanine, and

Thymine. Bases form pairs, either as  $A - T$  or  $C - G$ , that are ordered sequentially to form two long polymers, with backbones that run in opposite directions giving rise to a double-helix structure [242]. Arbitrarily, one of the polymers is called the positive strand and the other is called the negative strand.

Proteins are composed of chains of amino acids and, as explained by the central dogma of biology [6], DNA is the template that instructs the cellular machinery how to produce proteins. There are 20 amino acids, which are the building blocks of all proteins. Each of the twenty amino acids is encoded by a group of three DNA bases called a “codon” [58]. More than one codon can code for the same amino acid (i.e.  $4^3 = 64$  codons  $> 20$  amino acids) allowing for code redundancy. Additionally, there are codons that mark the end of the protein, these are called “STOP” and signal molecular machinery to end the translation process [26].

Proteins compose up to 50% of a cell’s dry weight compared to DNA which makes up only 3% [6]. Proteins perform their functions mainly by interacting with other proteins, forming complex pathways that execute a vast array of cellular functions including catalysing of chemical reactions, cell signalling, and providing structural conformation of the cell [6]. The 3-dimensional structure of a protein, also called “tertiary structure”, is tailored to bind to other proteins in a specific manner to accomplish a specific function.

The human genome has a total of 3 Giga-base-pairs (Gb), and those bases are divided into 22 autosomal chromosome pairs (in each pair one chromosome is maternally inherited and the other paternally inherited) and two sex chromosomes. The longest of the autosomal chromosomes is roughly 250 Mega-bases (MB) in length and the shortest one is 50 Mb.

In order to compare DNA from different individuals (or samples), we need a “reference genome”. Having a standard reference sequence facilitates comparisons and analysis. For most well studied organisms, reference genome sequences are available and current large scale sequencing projects are extending significantly the number of genomes known, e.g. one project seeks to sequence 10,000 mammalian genomes [104], another is targeting all microbes

that live within the human gut [231]. The human reference genome (e.g. GRCh37) does not correspond to the DNA of any particular person, but to a mosaic of the genomes of thirteen anonymous volunteers from Buffalo, New York [211].

When the genome of an individual is sequenced, the DNA is compared to the reference genome. Most of the DNA is the same, but there are differences. These differences, generically known as “genetic variants” (or “variants”, for short), describe the particular genetic make-up of each individual. There are several different ways a sample can differ from a reference genome. Each variant is the result of a mutations that happened at some point in the evolutionary history of the individual (or that of the reference genome). Variant types can be roughly categorized in the following way:

**Single nucleotide variants (SNV)** or Single nucleotide polymorphisms (SNP) are the simplest and more common variants produced by single base difference (e.g. a base in the reference genome, at a given coordinate, is an ‘A’, whereas the sample is ‘C’). Depending on whether the variant was identified in an individual or in a population, it is called a Single Nucleotide Variant (SNV) or Single Nucleotide Polymorphism (SNP). It is estimated that there are roughly  $3.6M$  SNPs per individual [47]. There are several biological mechanisms responsible for this type of variant: i) replication errors, ii) errors introduced by DNA repair mechanism, iii) deamination (a base is changed by hydrolysis which may not be corrected by DNA repair mechanisms), iv) tautomerism (and alteration on the hydrogen bond that results in an incorrect pairing) [98].

**Multiple nucleotide polymorphisms (MNP)** are sequence differences affecting several consecutive nucleotides and are typically treated as a single variant locus if they are in perfect linkage disequilibrium (e.g. reference is ACC whereas the sample is TGC).

**Insertions (INS)** refer to a sample having one or more extra base(s) compared to the reference genome (e.g. the reference sequence is AT and the sample is ACT). Short

insertions and deletions (indels) of a chromosome region range from 1 to 20 bases in length are reported to be 10 to 30 times less frequent than SNV [47]. Small insertions are usually attributed to DNA polymerase slipping and replicating the same bases (this produces a type of insertion known as duplication). Large insertions can be caused by unequal cross-over event (during meiosis) or transposable elements.

**Deletions (DEL)** are the opposite of insertions, the sample has one or more base(s) removed with respect to the reference genome (e.g. reference is ACT and sample is AT). As in the case of insertions, deletions can also be caused by ribosomal slippage, cross-over events during meiosis. Those include large deletions, which can result in the loss of an exon or one or more whole genes [6]. Short deletions are 10 to 30 times less frequent than SNV [47].

**Copy number variations (CNVs)** arise when the sample has two or more copies of the same genomic region (e.g. a whole gene that has been duplicated or triplicated) or conversely, when the sample has fewer copies than the reference genome. Copy number variations are often attributed to homologous recombination events [6].

**Rearrangements** such as inversions and translocations are events that involve two or more genomic breakpoints and a reorganization of genomic segments, possibly resulting in gene fusions or loss of critical regulatory elements. Inversions, a type of rearrangement, result from a whole genomic region being inverted.

As humans have two copies of each autosome, variants could affect zero, one or two of the chromosomes and are called “homozygous reference”, “heterozygous”, and “homozygous alternative” respectively. Variants are also classified based on how common they are within the population: common ( $\geq 5\%$ ), low frequency ( $\leq 5\%$ ), or rare ( $\leq 1\%$ ). How these types of genetic variants influence traits or disease risk is a topic of intense research that is discussed throughout this thesis.

## 1.3 DNA and disease risk

It would be fair to say that the Garrod family was fascinated by urine. As a physician at King’s College, Alfred Baring Garrod, discovered gout related abnormalities in uric acid [125]. His son, Sir Archibald Garrod, was interested in a condition known as alkaptonuria, in which children are mostly asymptomatic except for producing brown or black urine, but by the age of 30 individuals develop pain in joints of the spine, hips and knees. In 1902, Archibald observed that the family inheritance pattern of alkaptonuria resembled Mendel’s recessive pattern and postulated that a mutation in a metabolic gene was responsible for the disease. Publishing his finding he gave birth to a new field of study known as “Human biochemical genetics” [125].

Diseases having simple inheritance patterns, such as alkaptonuria, cystic fibrosis, phenylketonuria and Huntington’s are also known as Mendelian diseases [125]. The genetic components of several Mendelian diseases have been discovered since the mechanism was first elucidated by Garrod in 1902 and the process has been accelerated in recent years, thanks to the application of DNA sequencing techniques [14].

In complex diseases (or complex traits), such as diabetes or Alzheimer’s disease, affected individuals cannot be segregated within pedigrees (i.e. no simple pattern of inheritance can be identified). In contrast to Mendelian diseases the aetiology of complex traits is complicated due to factors such as: incomplete penetrance (symptoms are not always present in individuals who have the disease-causing mutation) and genetic heterogeneity (caused by any of a large number of alleles). This makes it more difficult to pinpoint the genetic variants that increase risk of complex disease as demonstrated by the failure of linkage analysis methods and later on GWAS [23].

### 1.3.1 Heritability and Missing heritability

We all know that “tall parents tend to have tall children”, which is an informal way to say that height is a highly heritable trait. It is said that there are 30 cm from the tallest 5% to the shortest 5% of the population and genetics account for 80% to 90% of this variation [250], which means that 27cm of variance are assumed to be “carried” by DNA variants from parents to offspring. Since 2010 the GIANT consortia has been investigating the genetic component of complex traits like height, body mass index (BMI) and waist to hip ratio (WHR). Even though they found many variants associated those traits, their findings only explain 10% of the phenotypic variance which corresponds to only a few centimetres in height [250].

In order to measure this genetic contribution to disease or traits we need a formal definition. Heritability is defined as the proportion of phenotypic variance that is attributed to genetic variation. The total phenotypic variation is assumed to be caused by a combination of “environmental” and genetic variations  $Var[P] = Var[G] + Var[E] + 2Cov[G, E]$  [261].

The environmental variance  $Var[E]$  is the phenotypic variance attributable only to environment, that is the variance for individuals having the same genome  $Var[E] = Var[P|G]$ . This can be estimated by studying monozygotic and dizygotic twins.

If the covariance factor  $Cov[G, E]$  is assumed to be zero, we can define heritability as  $H^2 = \frac{Var[G]}{Var[P]}$ . This is called “broad sense heritability” because  $Var[G]$  takes into account all possible forms of genetic variance:  $Var[G] = Var[G_A] + Var[G_D] + Var[G_I]$ , where  $Var[G_A]$  is the additive variance,  $Var[G_D]$  is the variance from dominant alleles, and  $Var[G_I]$  is the variance from interacting alleles (epistasis). Non-additive terms are difficult to estimate, so a simpler form of heritability called “narrow sense heritability” that only takes into account additive variance is defined as  $h^2 = \frac{Var[G_A]}{Var[P]}$  [261].

Focusing on narrow sense heritability, the concept of “explained heritability” is defined as the part of heritability due to known variants with respect to phenotypic variation ( $\pi_{explained} = h_{known}^2/h_{all}^2$ ). Similarly, missing heritability is defined as  $\pi_{missing} =$

$1 - \pi_{explained} = 1 - h_{known}^2/h_{all}^2$ . When all variants associated with traits are known, then  $h_{known}^2 = h_{all}^2$  and  $\pi_{missing} = 0$ .

Until recently, it was widely assumed by the research community that the problem of missing heritability lay in finding the appropriate genetic variants to account for the numerator of the equation ( $h_{known}^2$ ) [261]. However, in a series of theorems published recently, it has been proposed that there is a problem in the way the denominator is estimated [261]. In the aforementioned papers, Zuk et al. created a limiting pathway model ( $LP(k)$ ) that accounts for epistasis (gene-gene interactions) in  $k$  biological pathways. They showed that a severe inflation of  $h_{all}^2$  estimators occurs even for small values of  $k$  (e.g.  $k \in [2, 10]$ ). As a result, genetic variants estimated to account only for 20% of heritability, could actually account for as much as 80% using an appropriate model [261].

Even though this result is encouraging, the problem is now shifted to detecting epistatic interactions, a problem that we discuss this in section 1.7 and Chapter 4. Identifying epistatic interactions is a hard problem and requires very large sample sizes. In the same work [261], the authors show an example of power calculation assuming a single loci genetic effect that would require sequencing roughly 5,000 individuals to detect links to a genetic variant, which is a large but nowadays not uncommon, sample size. According to their estimates, finding an epistatic interaction of similar effect would require sample sizes as high as 500,000 individuals [261]. Even though this represents an extremely large number of samples, it is quickly becoming possible thanks to large technological advances and cost reductions in sequencing and genotyping technologies.

### 1.3.2 Conclusions

Although some genetic causes for complex traits, such as type II diabetes, have been found, only a small portion of the phenotypic variance can be explained. This might indicate that many risk variants are yet to be discovered. Recent studies on the topic of missing heritability suggest that the root of these “difficult to find genetic variants” might be found in

epistatic interactions (analysed in section 1.7.7) or rare variants (see section 1.6.6). Analysis of either requires more complex statistical models and larger sample sizes with the corresponding increase in computational requirements. In Chapter 4 of this thesis, we focus on methods for finding epistatic interactions related to complex disease and develop computationally tractable algorithms that can process data from sequencing experiments involving large number of samples in a reasonable amount of time.

## 1.4 Identification of genetic variants

Two of the main milestones in genetics were the discovery of the DNA structure in 1953 [242], followed by the first draft of the human genome in 2004 [45]. The cost of sequencing the first human reference genome was around \$3 billion (unadjusted US dollars) and it was an endeavour that took around 10 years. Since that time, DNA sequencing technology has evolved substantially so that a human genome can now be sequenced in three days for a price of less than \$1,000, according to prices estimated by Illumina, one of the main genome sequencer manufacturers [105].

The amount of information delivered by sequencing devices is growing faster than computer speed (Moore’s law) and data storage capacity [209]. A crude example, a leading edge sequencing system is advertised to be capable of delivering 18,000 human genomes at  $30\times$  coverage per year, yielding over 3.2 PB of information. Having to process huge amounts of sequencing information poses several challenges, a problem informally known as “data deluge”. From this raw data we want to obtain a set of candidate genomic variants that contribute to disease risk with the ultimate goal to translate these risk variants into biological knowledge. As expected, processing huge datasets consisting of thousands of sample is a complex problem. In Chapter 2 we show how to mitigate or solve some of these issues, by designing a computer language specially tailored to tackle what are known as “Big data” problems.

### 1.4.1 Sequencing data

DNA sequencing machines are based on different technologies, in a nutshell all these technologies detect a set of polymers (or chains) of DNA nucleotides and outputs a set of strings of A, C, G, and Ts. Unfortunately, current technological limitations make it impossible to “read” a full chromosome as one long DNA sequence. Instead, modern sequencers produce a large number of “short reads”, which range from 100 bases to 20 Kilo-bases (Kb) in length, depending on the technology [189]. Since sequencers are unable to read long DNA chains, preparing the DNA for sequencing involves fragmenting it into small pieces. These DNA fragments are a random sub-samples of the original chromosomes [214]. Reading each part of the genome several times increases accuracy and ensures that the sequencer reads as much as possible of the original chromosomes. The coverage of a sequencing experiment is defined as the number of times each base of the genome is read on average [214, 189]. For instance, if the sequencing experiment is designed to produce one billion reads, and each read is 150 bases long, then the total number of bases read is 150Gb. Since the human genome is 3Gb, the coverage is said to be 50.

After sequencing a sample, we have millions of reads but we do not know where these reads originate from in the genome. This is solved by aligning (also called mapping) reads to the reference genome, which is assumed to be very similar to the genome being sequenced. Once the reads are mapped, we can infer if the sample’s DNA has any differences with respect to the reference genome, a problem is known as “variant calling”.

Although sequencing costs are dropping fast, it is still expensive to sequence thousands of samples and in some cases it makes sense to focus on specific areas of the genome. A popular experimental set-up is to focus on coding regions (exons). A technique called “exome sequencing” [43] consists of capturing exons using a DNA chip and then sequencing the captured DNA fragments only. Exons are roughly 1.2% of the genome, thus this technique reduces sequencing costs significantly, for which it has been widely used by many research groups although it has the disadvantage of only analysing coding genomic variation.

## 1.4.2 Read mapping

Once the samples have been sequenced, we have a set of reads from the sequencer. The first step in the analysis is finding the location in the reference genome where each read is supposed to originate from, a process that is complicated by several factors: i) there are differences between the reference genome and the sample genome, ii) sequencing reads may contain errors, iii) several parts of the reference genome are quite similar making reads from those regions indistinguishable, and iv) a typical sequencing experiment generates millions of reads [214].

**Local sequence alignment** We introduce a problem known as *local sequence alignment*: Given two sequences  $s_1$  and  $s_2$  from an alphabet (e.g.  $\Sigma = \{A, C, G, T\}$ ), the alignment problem is to add gap characters ('-') to both sequences, so that a distance measure, such as Levenshtein distance,  $d(s_1, s_2)$  is minimized. This problem has a well known solution, the Smith-Waterman algorithm [219], which is a variation of the global sequence alignment solution from Needleman-Wunsch [168], and has an algorithm complexity  $O(l_1.l_2)$  where  $l_1$  and  $l_2$  are the length of the sequences. So, Smith-Waterman algorithm is slow since in this case one of the sequences is the entire genome.

In order to speed up sequence alignment, several heuristic approaches emerged. Most notably, BLAST [7], which can be used to map sequences to a reference genome. BLAST uses an index of the genome to map parts of the query sequence, called seeds, to the reference genome. Once these seeds have been positioned against the reference, BLAST joins the seeds performing an alignment only using a small part of the reference.

**Read mapping** Sequence alignment has an exact algorithmic solution and several faster heuristic solutions. But even the fastest solutions are too slow to be used with the millions of reads generated in a typical sequencing experiment. Faster algorithms can be used if we relax our requirements in two ways: i) we allow for sub-optimal results, and ii) instead of requiring

the output to be a complete local alignment between a read and the genome, we just want to know the region in the reference genome where the read sequence is from. This relaxed version of the alignment algorithm is called “read mapping” and the reduced complexity is enough to speed up the computations significantly. In a nutshell, a read mapping is regarded as correct if it overlaps the true reference genome region where the read originated. Once the mapping is performed, the read is locally aligned, a strategy similar to BLAST algorithm [144, 137].

Reformulating the alignment problem as a *mapping* problem allows us to use data structures such as suffix trees to index the reference genome. Using suffix trees we can query for a substring (read) [74] of the indexed string in  $O(m)$  time, where  $m$  is the length of the query. Alternatively, we can use suffix arrays which provide a space optimized alternative to suffix trees [74]. An implicit assumption in this solution, is that the read is very similar to the reference and that there are no gaps. Suffix arrays algorithms are fast but, even though they are memory optimized versions of suffix trees, memory requirements are still high ( $O[n \log(n)]$ , where  $n$  is the length of the indexed sequence, in this case the reference genome) and this becomes the limiting factor. In order to reduce the memory footprint of suffix arrays, Ferragina and Manzini [81] created a data structure based on the Burrows-Wheeler transform. This structure, known as an FM-Index, is memory efficient yet fast enough to allow mapping of a high number of reads. An FM-index for the human genome can be built in only 1Gb of memory, compared to 12Gb required for an equivalent suffix array [144]. Given a genome  $G$  and a read  $R$ , an FM-index search can find the  $N_{occ}$  exact occurrences of  $R$  in  $G$  in  $O(|R| + N_{occ})$  time, where  $|R|$  is the length of  $R$  [144].

We should keep in mind that suffix trees, suffix arrays and FM-indexes are guaranteed to find all matching substring occurrences, nevertheless a sequencing read may not be an exact substring of the reference genome (due to sample’s genome differences with the reference genome, read errors, etc.). So, even if efficient indexing and heuristic algorithms can decrease mapping time considerably, these algorithms are not guaranteed to find an optimal

mapping. Several parameters, such as the read length, sequencing error profile, and genome complexity profile can affect their performance. The most commonly used implementation of the FM-index mapping algorithms are BWA [144, 145] and Bowtie [137, 136]. Each provides optimized versions for the two most common sequencing types: i) short reads with high accuracy [144, 137] or ii) longer reads with lower accuracy [145, 136]. It should also be taken into account that read-mapping algorithms implement heuristics to map reads having differences with respect to the reference genome. Obviously these heuristics are implementation dependent, thus two mapping algorithms can (and often do) lead to different mappings for the same read set which in turn can lead to different variants being called (see section 1.4.3).

**Mapping quality** Sequencers not only provide sequence information, but also provide an error estimate for each base [143]. This is often referred as a quality ( $Q$ ) value, which is the probability of an error, measured in negative decibels  $Q = -10 \log_{10}(\epsilon)$ , where  $\epsilon$  is the error probability. Mapping quality is an estimate of the probability that a read is incorrectly mapped to the reference genome.

Mapping algorithms provide estimates of mapping quality. In the MAQ model [146], which is one of the earliest models for calculating mapping quality, three main sources of error are explored: i) the probability that a read does not originate from the reference genome (e.g. sample contamination); ii) the probability that the true position is missed by the algorithm (e.g. mapping error); and iii) the probability that the mapping position is not the true one (e.g. if we have several possible mapping positions). It is assumed that the total error probability can be approximated as  $\epsilon \approx \max(\epsilon_1, \epsilon_2, \epsilon_3)$ .

### 1.4.3 Variant calling

Genome-wide variant calling has until recently largely been done using genotyping arrays (for SNVs) or Comparative Genomic Hybridization arrays (for CNVs). The inherent limitations of these technologies, particularly their ability to only assay genotypes at sites that are

known in advance to be polymorphic, combined with the declining cost of sequencing, have now made approaches based on high-throughput resequencing the tool of choice for variant calling in clinical studies.

Once the sequencing reads have been mapped to the reference genome, we can try to find differences between a sequenced sample and the reference genome. This process is called “variant calling” [170]. Several factors complicate this task, the two main ones being sequencing errors and mapping errors, described in 1.4.2. Based on sequencing data and mapping error estimates, tools such as GATK [160] and SamTools/BcfTools [146] use maximum likelihood models to infer when there is a mismatch between the sample and reference genomes and whether the sample is homozygous or heterozygous for the variant. This method works best for differences of a single base (SNV), but it can also work with different degrees of success for short insertions or deletions (InDels) consisting of less than 10 bases.

Aligning sequences that contain InDels (gaps) is more difficult than ungapped alignments since finding the optimal gap boundary depends on the scoring method being used. This biases variant calling algorithms towards detecting false SNVs near InDels [69]. An approach to reduce this problem is to look for candidate InDels and perform a local realignment in those regions, to reduce significantly the number of false positive SNVs [69]. Another approach involves estimating a “Base Alignment Quality” (BAQ) [142] score, which is the probability of misalignment for each base. It can be shown that replacing the original base quality with the minimum between base quality and BAQ produces an improvement in SNV calling accuracy. The BAQ can be calculated using a special type of “Hidden Markov Model” (HMM) designed for sequence alignment [142, 74]. A more sophisticated option for reducing errors consist of performing a local genome re-assembly on each polymorphic region (e.g. HaplotypeCaller algorithm [225]).

Finally, one should note that the error probabilities inferred by the sequencers are far from perfect. Once the variants have been called, empirical error probabilities can be easily calculated [160] by comparing sequenced variants to a set of “gold standard variants” (i.e.

variants that have been extensively validated). This allows to re-calibrate or re-estimate the error profile of the reads. This is known as a re-calibration step, and usually improves the number of false positives calls [69].

Due to the nature of short reads, this family of methods does not work for structural genomic variants, such as large insertions, deletions, copy number variations, inversions, or translocations. A different family of algorithms are used to identify structural variants generally making use of pair end reads or split reads, but their accuracy so far has been low compared to SNV calling algorithm [174].

One of the caveats of current sequencing technologies and computational methods for variant calling is that detection accuracy varies significantly for different variant types. SNVs are by far the most accurately detected. Insertions and deletions, collectively referred as InDels, can be detected less efficiently depending on their sizes. Small InDels consisting of ten bases or less are easier to detect than large InDels consisting of 200 bases or more [75], in part as most commonly used sequencers reads DNA in stretches roughly 200 bases long. Due to this technological limitation, detection is less reliable for more complex variant types.

## 1.5 Functional annotations of genomic variants

The development of cost-effective, high-throughput sequencing technologies have had a profound impact on our ability to study the effects of individual genetic variants on the pathogenesis and progression of both monogenic and common polygenic diseases. As sequencing costs decrease and throughput increases, it has now become possible to quickly identify a large number of sequence polymorphisms (SNVs, indels, structural) using samples from affected and unaffected subjects and investigate these in epidemiologic studies to identify genomic regions where mutations increase disease risk. However, translating this information into biological or clinical insights is challenging as it is often difficult to determine which specific polymorphisms are the main pathogenetic drivers of disease across a population; and

more importantly, how they affect the activity of disease-related molecular pathways in tissues and organism a specific patient. In part, this difficulty results from the large number of genetic variants that are observed in individual genomes (the human population is believed to contain approximately 3.5 million polymorphic sites with minor allele frequency above 5%) combined with the limited ability of computational approaches to distinguish variants with no impact on genome function (the vast majority) from variants affecting gene function or expression that may be associated with disease risk or drug response (the minority). The development of algorithms for automated variant annotation, which link each variant with information that may help predict its molecular and phenotypic impact, is a critical step towards prioritizing variants that may have a functional impact from those that are harmless or have irrelevant functional effects. In this section we review the key concepts and existing approaches in this important field. In Chapter 3 we introduce an approach to collect relevant information that will help answer questions about genetic variants discovered in sequencing studies, including: (i) will a given coding variant affect the ability of a protein to carry its functions; (ii) will a given non-coding variant affect the expression or processing of a given gene; and ultimately (iii) will a given coding or non-coding variant have any impact on phenotypes of interest?

Answering these questions is essential for many types of analyses that use large-scale genomics datasets to study quantitative traits and diseases, particularly when only a small number of individuals is studied comprehensively at a genome-wide level. For example, most genome-wide association studies (GWAS) or exome sequencing studies lack the statistical power to identify rare variants or variants with small effects associated with a disease, in part due to the large number of variants assayed. This limitation can be addressed by directing both statistical analysis and subsequent experimental steps to focus on smaller sets of genetic variants that have been prioritized based on external evidence of their putative impact. The common impairment of DNA repair mechanisms and chromatin stability in malignant cells leads to a similar challenge in cancer genomics, where the hundreds or thousands of mutations

that distinguish an individual’s tumor and germline genomes need to be classified on the basis of their putative phenotypic effects and potential roles in carcinogenesis.

The large number of databases containing potentially helpful information about a given variant make the process of gathering and presenting relevant data challenging, despite excellent tools that already exist to analyse large genomics datasets (including GATK [160] and Galaxy [93]) and visualize the results (such as the UCSC [123] or Ensembl [85] genome browsers). Each of these databases uses its own format and is updated asynchronously, which makes it difficult for any analysis to remain up to date. In addition, the lack of comprehensive and computationally efficient models that allow integrative analyses using these resources, makes the task of comprehensive variant annotation overwhelming. By efficiently combining information from tens or hundreds of genome-wide databases, the tools described here are designed to greatly facilitate the process of variant annotation, and make it accessible to groups with limited bioinformatics expertise or resources.

### 1.5.1 Variant types

Although variant calling is a challenging task and remains an important area of research, many high-quality tools exist for calling SNVs and indels. We discuss here the problem of annotating the variants identified by some of these tools. The most common type of variant identified by current technologies and analysis approaches is a single base difference with respect to the reference genome (SNV) followed by multiple base differences (MNP), as well as small insertions and deletions (InDels). Here, we focus on annotating these three types of variants which comprise most of the variants in a typical sequencing experiment. We do not address the annotation of large rearrangements due to the challenges involved in their identification and functional characterization and their relative rarity in the germ line.

### 1.5.2 Types of genetic annotations

The process of genetic variant annotation consists of the collection, integration, and presentation of experimental and computational evidence that may shed light on the impact of each variant on gene or protein activity and ultimately on disease risk or other phenotypes. Variant annotation has traditionally been divided in two apparently independent but actually interrelated tasks based on the variant's location with respect to known protein-coding genes. Coding variant annotation focuses on variants that are located within coding regions of annotated protein-coding genes and attempts to assess their impact on protein function. In contrast, non-coding variant annotation focuses on variants located outside the coding portion of genes (i.e. in intergenic regions, UTRs, introns, or non-protein-coding genes) and aims to assess their potential impact on transcriptional and post-transcriptional gene regulation. These two categories of variant annotations are not mutually exclusive, as variants located within exons can often have an impact on the gene transcript's processing (splicing). In addition, some transcripts can have function as a result of both their protein-coding and non-coding potential [6]. Despite the intermingling of the notion of coding and non-coding variants, we will consider each type of annotation separately as assessing their impact requires different sources of data and algorithms.

The ultimate goal of variant annotation is to predict the impact of a sequence variant, although this is an ill-defined term. On the one hand, one may be interested in the molecular impact of a variant on the activity of a protein. On the other, one may be interested in a variant's impact on much higher-level phenotypes such as disease risk. Mutations that are predicted to completely abrogate a gene's activity are called loss-of-function (LOF) mutations. Those that are predicted to have less severe consequences are called moderate or low impact mutations. In practice, a variant will be predicted to cause LOF if it has two properties: i) its molecular impact is reliably predictable by existing computational approaches (e.g. gain of stop-codon); and ii) its functional impact, reflected by altered protein activity or expression levels, is expected to be large. Many types of variants, including

most non-coding variants, may have a large functional impact but lack predictability, and as a consequence are typically not predicted to be LOF variants.

### 1.5.3 Coding variant annotation

Coding variants occur in translated exons. When a reliable gene annotation is available, their main impact can be classified by determining their effect on the translated amino acid sequence (if any). A synonymous coding variant (also called silent) does not change the sequence of amino acids encoded by the gene, although it may impact aspects of post-transcriptional regulation such as splicing and translation efficiency and can affect the total protein activity through changes in the amount of translated protein that is made in the cell. In contrast, a non-synonymous coding variant changes one or more amino acids encoded by the gene and can directly alter the protein's activity, localization or stability. Non-synonymous variants include missense substitutions that change a single amino acid, nonsense substitutions that lead to the gain of a stop codon, frame-preserving indels that insert or delete one or more amino acids, and frame-shifting indels that may completely alter the protein's amino acid sequence. Primary annotation and assessment of impact, determines whether a variant falls in any of these categories.

Several factors can affect prediction accuracy in coding variant annotations:

- i *Gene misannotation.* Genomic variants that have a significant effect on a protein's expression or function represent a very small fraction of all variants. Assembly and gene annotation errors or genomic oddities that break classical computational models are also rare, but lead to false positives. This implies that one is likely to find a non-negligible fraction of false-positive high-impact variants among the list of what appear to be the strongest candidates for variants with severe effects. Tools such as SnpEff can anticipate some of the most common causes of misannotation, but the number and diversity of the type of events that can lead to false-positives makes the task very challenging. As a consequence, one should always manually inspect the top candidates

to ensure that they have been assigned to the correct genes and transcripts.

- ii *Gene isoforms.* In higher eukaryotes, most genes have more than one transcript (or isoform), due to alternative promoters, splicing, or polyadenylation sites. For example, a human gene has an average of 8.8 annotated messenger RNA (mRNA) isoforms and some genes are believed to have over 4,000 isoforms resulting from complex splicing programs. For these genes, a variant may be coding with respect to one mRNA isoform and non-coding with respect to another. There are two frequent approaches to address this situation: (i) annotate a variant using the most severe functional effect predicted for at least one mRNA isoform; or (ii) use only a single canonical transcript per gene to perform primary annotation.
- iii *Variant calling for indels.* Variant annotation relies on knowing the exact genomic coordinates of the variant: this is rarely a problem for isolated SNVs; however, insertions and deletions often cannot be located unambiguously. Consider for example the variant  $AA \rightarrow A$ . This mutation results in the loss of a single base, but was it the first or second A that was deleted? From the standpoint of the cell, this question is irrelevant and deletion of any A will have the same effect. In contrast, from the standpoint of most variant annotation software, deleting the first A is different from deleting the second. Consider the scenario of a previously annotated transcript where the first A is part of the 5' UTR and the second is the first base of a start codon. If the missing base is assigned to the leftmost position in the motif (as is the current convention), the deletion would be annotated as a low impact 5'UTR variant. However, assigning it to the rightmost A would make it appear to be a high-impact start-codon deletion. Similar issues may arise when considering conservation scores or transcription factor binding site (TFBS) predictions.

#### 1.5.4 Loss of function variants

True LOF variants are difficult to predict computationally, but specific types of genetic changes will frequently lead to severely impaired protein activity. These include i) stop-gains, also known as nonsense mutations; ii) start-loss mutations which change or remove the transcript's start codon; iii) indels causing frame-shifts; iv) large deletions that remove either the first exon or at least 50% of the protein coding sequence; and v) loss of splice acceptor or donor sites that alter the protein-coding sequence. Variants that introduce premature in-frame stop codons (nonsense mutations and most frame-shift indels) are expected to abolish protein function, unless the variant is very near the C-terminus of the coding region [252] (effectively, downstream of the last functional domain in the protein). Such mutations may have severe consequences in affected cells, tissues or organism, as is seen for mutations that cause monogenic diseases [210]. In addition, a new stop codon that lies upstream of the last exon will likely trigger nonsense mediated decay (NMD), a process that degrades mRNA before protein synthesis occurs [166]. NMD predictions are not exact and many factors can affect mRNA degradation, including the variant's distance from the last exon-exon junction or poly-A tail, and the possibility that transcription may re-initiate downstream of the LOF variant [27].

A variant that leads to the loss of a stop codon, sometimes called a read-through mutation, will result in an elongated protein-coding transcript that terminates at the next in-frame stop codon. While there are no general models that predict how deleterious this may be, such variants can also result in aberrant folding and degradation of the nascent proteins, leading to activation of cellular stress response pathways, in addition to their direct effects on protein activity and expression levels [210].

The effect of the loss of a start codon depends on the location of a replacement start codon with respect to the translation start site and reading frame of the native protein. If the new start codon maintains the reading frame, the only consequence may be the loss of a few amino acids in the protein transcript; however, in many cases, the new start codon will

not be in-frame, thus producing a frame-shifted protein that is later degraded. In addition, the new start codon may lack an appropriate regulatory context (for example, if there is no Kozak sequence nearby or if it disrupts 5' UTR folding) leading to reduced expression of an N-terminally truncated protein. Consequently, losing a start codon is thought to be highly deleterious in most cases, due to the potential that it may reduce both protein production and activity.

Variants affecting rare amino acids are also thought to cause loss of function in proteins. Through a process called translational recoding, a UGA “Stop” codon located in the appropriate mRNA context (determined by both primary mRNA sequence and secondary structure) may be translated to incorporate a selenocysteine amino acid (Sec / U) [6]. In humans, it is known to occur 100 codons located in mRNAs whose 3' UTR contains a Selenocysteine insertion sequence element (SECIS). Since the translation machinery goes so far to encode these special rare amino acids, the expectation is that mutations at those sites would be highly deleterious. This is supported by evidence that reduced efficiency of selenocysteine incorporation is linked to severe clinical outcomes, such as early onset myopathy [153] and progressive cerebral atrophy [4].

Since algorithms rely on heuristics, LOF predictions are affected by false-positives, variants predicted to result in a LOF sometimes actually produce proteins that are partially functional [152]. In fact, an apparently healthy individual is typically heterozygous for around 100 predicted LOF variants, and homozygous for roughly 10, but many of those are unlikely to completely abolish the protein function. Indeed, these variants are enriched toward the 3' end of the gene, where they are likely to be less deleterious.

### 1.5.5 Variants with low or moderate impact

Compared to the high impact variants discussed above, where extensive prior biological evidence strongly suggests that a specific type of variant will severely impair protein activity, there are few guidelines that can reliably predict how the majority of non-synonymous (mis-

sense) variants will alter protein function or expression. As a result, the primary annotation performed by SnpEff and most related software packages will broadly categorize missense substitutions and their accompanying amino acid changes (e.g.  $K154 \rightarrow L154$ ) as moderate impact variants. Short indels whose length is a multiple of three are treated similarly, unless they introduce a stop codon, as their effect will usually be localized.

Once missense and frame-preserving InDel variants are identified, a more detailed estimation of their impact on protein function can be performed using heuristic and statistical models. The most common approaches are based on sequence conservation, either amongst orthologous or homologous proteins, or protein domains, sometimes adding information of the physio-chemical properties of the reference and variant amino acids (e.g. differences in side chain charge, hydrophobicity, or size). The SIFT algorithm [133] assesses the degree of selection against specific amino acid changes at a given position of a protein sequence by analysing the substitution process at that site throughout a collection of predicted homologous proteins identified by PSI-BLAST [8]. Based on this multiple sequence alignment and the highly conserved regions it contains, SIFT calculates a normalized probability of amino acid replacement (called the SIFT score), which estimates the mutation’s effect on protein function. Polyphen [3], another commonly used tool, takes the process one step further by searching UniProtKB/Swiss-Prot [50] and the DSSP database of secondary structure assignments [121] to determine if the variant is located in a known active site in the protein. In contrast to other methods that categorize each variant individually, VAAST [200], a commercially available package, computes scores for groups of variants located within a given gene and “collapses” them into a single category, a concept similar to burden testing performed for rare variants identified in exome sequencing studies. For human proteins, SnpEff makes use of the Database for Non-synonymous SNVs’ Functional Predictions [150] (dbNSFP), which collects scores produced by several impact assessment algorithms in a single database. Individually, impact assessment methods usually have an estimated accuracy of 60% to 80% when compared to manually curated databases of human variants, but pre-

dictions from several algorithms can be combined to provide a stringent, but more accurate estimate of impact [37].

In most cases these algorithms apply best to SNVs since these are common in populations and there is more genomic sequence and experimental data available to refine the statistical methods. However, some recently developed algorithms are capable of assessing variants other than SNVs, including PROVEAN [37], which extends SIFT to assess the functional impact of indels.

Non-synonymous and synonymous variants are difficult to assess due to several factors:

- i *Imprecise models of protein function.* Accurate impact assessment of coding variants remains an open problem and most computational predictions are riddled with both false positives and false negatives. While both missense variants and frame-preserving indels are broadly catalogued as having moderate effects, this is mostly due to lack of a comprehensive model and the extremely complex computations that would be required for an in-depth analysis (such as protein structure predictions). In these cases, proteomic information can be revealing. SnpEff adds annotations from curated proteomic databases, such as NextProt [135], which can help to elucidate if a mutation alters a critical protein amino acid or domain (such as amino acids that are post-translationally modified as part of a signalling cascade or that are form the active site of an enzyme) resulting in a protein may no longer function.
- ii *Gain of deleterious function.* Computational variant annotation may eventually be able to fairly accurately predict the molecular impact of a variant in terms of the degree to which it translates in a loss of function for the encoded protein. However, gains of function, including the acquired ability to interact with new partners and disrupt their function, remain vastly more difficult to tackle, although several such variants have been linked to disease [245].
- iii *Unanticipated effects of synonymous variants.* In most cases, synonymous variants are

regarded as non-deleterious (or low impact); however, one needs to seriously consider the possibility that they may have greater functional effects by altering mRNA splicing [55] or secondary structure [203]. Synonymous SNVs may also alter translation efficiency, by changing a frequently used to a rarely used codon and have been linked to changes in protein expression [206].

### 1.5.6 Non-coding variant annotation

Although coding variants represent less than 2% of variants in the human genome, they make up the vast majority of confirmed disease-related variants that have been validated at a functional level. This may result from ascertainment bias (since variants in coding regions are straightforward to discover and characterize at a basic level and many studies have largely ignored non-coding variants); or may be explained by the increased complexity of computational approaches and lab assays required to predict and validate the impact of non-coding variants; or by their potentially more subtle impact on gene expression or cell function. Nonetheless, in a compendium of current GWAS studies, roughly 40% of the variants are intergenic and 30% intronic. Functional studies of these variants are increasingly emphasizing the importance of non-coding genetic variation at risk loci for complex genetic diseases and traits [109].

Functional non-coding regions of the genome encompass a wide variety of regulatory elements contained in DNA and RNA molecules that are involved in transcriptional and post-transcriptional regulation. Cis-regulatory elements include (i) binding sites for DNA-binding proteins such as transcription factors and chromatin remodelers; (ii) binding sites for RNA-binding proteins involved in splicing, mRNA localization, or translational regulation; (iii) micro RNA (miRNA) target sites; and (iv) long non-coding RNA (lncRNA) targets on DNA, RNA and proteins. Non-coding transcripts include well-characterized regulatory RNAs (e.g. miRNA, snoRNA, snRNA, piRNA and lncRNAs) as well as RNAs involved directly in protein synthesis (e.g. tRNA and rRNA). The annotation and impact assessment

of non-coding variants presents a significant challenge for several reasons: (i) reliable technologies to study transcriptional regulatory regions on a genome-wide basis are only just reaching maturity and provide limited resolution of binding sites for individual transcription factors and regulatory RNA molecules; (ii) non-coding functional regions of most genomes remain incompletely mapped as they vary widely among different cell types and cell states (for example, in diseased and healthy tissues); (iii) non-coding regulatory elements often are part of complex transcriptional programs that are time-dependent [158], contain many redundant linkages or reciprocal connections between genes and respond to a wide range of intra and extracellular signals; and (iv) genomic regulatory elements rarely have a strict consensus sequence (for example, compare the position weight matrices used to identify transcription factor or miRNA binding sites with the amino acid triplet code) making the effect of a mutation on gene regulatory programs difficult to predict. As a result, high-quality annotation of non-coding variants relies more heavily on experimental data than is the case for coding variants: since many of these experimental techniques did not study the effects of SNVs on gene regulatory programs, they can only be used to annotate variants and not to predict their effects on gene transcription. In the few cases where the effects of SNVs have been studied (for example, the effects of SNVs that are common in a population and located in genetic loci associated with complex diseases), experimental approaches provide highly accurate functional assessment at a cost of reduced coverage compared to computational approaches.

Large-scale projects such as ENCODE [49] and modENCODE [33] have made major steps toward mapping gene transcription and transcriptional regulatory regions in many tissues and cell types, but similar studies in diseased tissues remain at an early stage (for example, the growing collection of disease-related epigenomes from the Epigenome Roadmap [19]). The base-by-base resolution and number of cell states studied for different types of regulatory elements and non-coding transcripts varies widely among datasets; in part due to the lack of sensitive, comprehensive and high-resolution technologies to study the different

molecular species and modes of interaction that can be altered by non-coding variants. Efficient technologies for genome-wide, high-throughput mapping of binding sites for RNA-binding proteins (PAR-CLIP [11]), miRNAs (PAR-CLIP [101] and CLASH [106]) are starting to be applied on a broad scale as are protocols to map transcription factor binding sites (TFBS) which can improve resolution to a single base (ChIP-exo [196]). However, in most cases, DNA and RNA binding sites are only imprecisely located within ChIP-Seq peaks that span genomic regions hundreds of base pairs in length, with computational approaches being used to pinpoint the bases most likely mediating the interaction. In the absence of more precise localization data, *de novo* computational prediction of binding sites for DNA and RNA binding proteins remains insufficiently accurate to be of much use in annotating single non-coding variants.

This limitation is particularly critical for functional predictions of putative target sites for microRNAs and other regulatory RNA species. MicroRNAs are short RNA molecules that regulate gene expression post-transcriptionally by binding the messenger RNA of a gene through complementary, usually in the 3' region of the transcript, which leads to mRNA degradation or inhibits translation. Sequence variants that cause the loss or gain of a miRNA target site would lead to dysregulation of the gene, with likely deleterious effects. Although miRNAs are relatively well documented in most model organisms including human, their binding sites are only starting to be mapped experimentally, and computational predictions has very low specificity. Meaningful information regarding the possible role of a variant in disrupting a miRNA target site is starting to emerge [148], although variants that create new miRNA binding sites remain under the radar.

Even if the position of a functional element could be perfectly determined, predicting a variant's impact on chromatin conformation, promoter activity, gene expression, or transcript processing remains challenging. For transcription factors, this involves predicting whether the protein will still be able to recognize its mutated site (and with what affinity), as well as predicting the impact of these changes on gene expression levels. The latter is particu-

larly hard to predict as a result of interactions, competition, and redundancy contained in regulatory networks of transcription factors or RNA binding proteins. As a consequence, computational prediction of the functional impact of non-coding variants remains a very active area of research and there is no broad consensus on the best methodology to use [241]. One significant exception is the identification of variants affecting canonical splice sites, defined as two bases on the 3' end on the intron (splice site acceptor) and 5' end of the intron (splice site donor). Variants that affect canonical splice sites are easily detected and typically lead to abnormal mRNA processing, involving exon loss or extension that leads to loss of function of the encoded protein.

### 1.5.7 Impact assessment of non-coding variants

Two broad classes of publicly available genome-wide datasets are commonly combined to assess the functional impact of non-coding genetic variants: (i) computational predictions of sequence conservation and sites involved in molecular interactions such as transcription factor and RBP binding, as well as miRNA-mRNA target interactions; and (ii) experimental genome-wide localization assays for DNA binding proteins, histone modifications, and chromatin accessibility.

**Computational sources of evidence** Interspecies sequence conservation plays a key role in scoring and prioritizing non-coding variants. This is based on the assumption that sites or regions that have been more conserved across species than expected under a neutral model of evolution are likely to be functional; suggesting that mutations contained in them are likely to be deleterious. In the absence of strong experimental data, sequence conservation measures calculated from whole genome multiple alignments, (for example using PhastCons [217], SciPhy [87], PhyloP [184], and GERP [65]), have been developed to provide a generic indicator of function for non-coding variants. Although high conservation scores generally mean that a genomic region may be functional, the converse is not true and many

experimentally-proven non-coding functional regions show only modest sequence conservation (for example due to binding site turnover events). Finally, some regulatory regions (e.g. specific elements regulating immune response [190]) are under positive selection and may thus show less conservation than surrounding neutral regions.

In humans, genome-wide computational predictions of transcription factor binding sites based on matching to publicly available position weight matrices are available from variety of sources, including Ensembl [85] and Jaspar [29]. Because of the low information content of most binding affinity profiles, the specificity of the predictions is generally very low. Related approaches exist to predict splicing regulatory regions [78] and miRNA target sites [260], some of which are precomputed for whole genomes and available from the UCSC or Ensembl genome browsers. Recent efforts to determine RNA-binding protein sequence affinities can also be used to identify putative binding sites for these proteins in mRNA [192].

**Experimental sources of evidence:** To investigate the potential impact of variants on transcriptional regulation, many published experimental data sets produced by large-scale projects such as ENCODE [49], modENCODE [33] and Roadmap Epigenomics [19], can be used directly by annotation packages. These include: (i) ChIP-seq or ChIP-exo experiments that identify TFBSS on a genome-wide basis; (ii) DNaseI hypersensitivity or Formaldehyde-Assisted Isolation of Regulatory Elements (FAIRE) assays that identify regions with open chromatin; and (iii) ChIP-seq studies to identify the presence of specific promoter or enhancer-associated histone post-translational modifications, which can be combined to identify active, poised, and inactive enhancers and promoters [192]. Most of these data sets are easily available through Galaxy [93] (as tracks from the UCSC Genome Browser) or through SnpEff (as downloadable pre-computed datasets). In parallel with the types of studies described above, expression quantitative trait loci (eQTLs) represent an agnostic way to map putative regulatory regions. An increasing number of such loci are available through the GTEx database [151]. Experimental data that may support assessment of the impact

of variants on post-transcriptional regulation remain sparser, although databases such as doRiNa [9] or starBase [253] contain genome-wide datasets obtained by CLIP-Seq and degradome sequencing. To our knowledge, these data have yet to be used in the context of variant annotation studies.

**Combining sources of evidence:** Despite the variety of computational and experimental sources of evidence available, impact assessment for non-coding variants remains relatively crude, due to the fact that biological models of gene regulation remain fairly simple. Nonetheless, significant steps forward have been made recently and two web-based tools, HaploReg [240] and RegulomeDb [24], perform SNV and indel impact assessment for variants from dbSNV on the basis of a broad body of computational and experimental evidence. Both use pre-computed scores for variants from dbSnp and therefore cannot be used for rare variants, but they are extremely valuable for exploration by associating the variant of interest with a variant in dbSnp via linkage disequilibrium.

Despite some advances in the field, non-coding variants remain one of the most challenging to analyse and annotate. Many factors add to the complexity of the problem, such as:

- i *Sparseness of functional sites within ChIP-seq peaks.* Even if a non-coding variant is located in a region that contains a ChIP-seq peak for a given TF and has all the hallmark signatures of regulatory chromatin, the likelihood that it is deleterious remains low, because most DNA bases contained within a peak are non-functional.
- ii *Gain of function mutations.* While this section has focused on variants causing the loss of a functional regulatory element, genetic variants may also create new or more effective transcription factor binding sites. These are substantially harder to detect as they can occur in regions that show no evidence of function in individuals possessing the reference allele, and show little conservation across species. Furthermore, computational methods to predict gain of affinity for a given TF caused by a variant have

insufficient specificity to be of much use on their own.

### 1.5.8 Clinical effect of variants

One of the most revealing types of annotation of both coding and non-coding variants reports whether the variant has previously been implicated in a phenotype or disease. Although such information is available for only a small minority of all deleterious variants, their number is growing and should be the first type of annotation one seeks out. Clinical annotations, until recently, have been scattered in a large number of specialized databases of medical conditions with a genetic basis, including the comprehensive, manually curated collection of genetic loci, variants and phenotypes in the Online Mendelian Inheritance in Man database [102] (OMIM, [www.omim.org](http://www.omim.org)); web pages containing detailed clinical and genetic information about uncommon disorders in the Swedish National Board of Health and Welfare Database for Rare Diseases ([www.socialstyrelsen.se/rarediseases](http://www.socialstyrelsen.se/rarediseases)) and the peer-reviewed NIH GeneReviews collection [29] ([www.ncbi.nlm.nih.gov/books/NBK1116](http://www.ncbi.nlm.nih.gov/books/NBK1116)); and a curated collection of over 140,000 mutations associated with common and rare genetic disorders in the commercial Human Gene Mutation Database [221] (HGMD, [www.hgmd.org/](http://www.hgmd.org/)). In most cases, these datasets do not use standardized data collection or reporting formats; are designed to primarily provide information to patients and health professionals through a web interface; and rely on heterogeneous criteria to describe disease phenotypes and clinical outcomes; pathological and other clinical laboratory data; as well as the genetic and biologic experiments that have been used to demonstrate disease mechanisms at a molecular or cellular level. These shortcomings are being addressed by initiatives that provide centralized, evidence-based, comprehensive collections of known relationships between human genetic variants and their phenotype that are suitable for computational analysis, such as the NIH effort to aggregate records from OMIM, GeneReviews [175] and locus-specific databases in ClinVar [134] ([www.ncbi.nlm.nih.gov/clinvar](http://www.ncbi.nlm.nih.gov/clinvar)).

Another important application of variant detection and annotation is in the study of

cancer genomes, which is occurring increasingly in clinical settings to support treatment decisions for advanced tumors. Annotation of variants detected in tumor sequences can be analysed for clinical cohorts, using similar techniques as other complex traits, as well as for individual patients, using techniques to identify differences between somatic (tumor) and germline (healthy) tissues. In the latter case, one looks for cancer-associated mutations that distinguish the somatic genome of cancer cells of an individual from the germline genome in order to find the driving mutations that pinpoint the specific mechanisms underlying tumorigenesis or metastasis. Ideally, these mutations can be used to select a treatment for the patient, establish prognosis, or to identify causative mutations that have led to the cancer’s progression. In such a setting, given that sequence differences between the cancer and germline genomes are of greater interest than the background genetic changes between the germline and a reference genome, variant calling is performed using specialized algorithms, such as MuTect [38] and SomaticSniper [139].

One of the main problems in these databases is annotation accuracy. Biological knowledge, as well as molecular and phenotypic evidence supports the identification of certain groups of high impact variants based on simple criteria (such as premature stops, frame-shifts, start lost and rare amino acid mutations); however, it is often hard to predict whether non-synonymous variants will have equally large effects on an organism’s health. Even when the accepted “rules of thumb” used in the primary annotation indicate that protein function is impaired, we should consider that these predictions may be based on a small number of model genes and will require appropriate wet-lab validation or confirmatory studies in cohorts. In addition, as more human genomes are sequenced, it is likely that some genetic variants that have been linked to Mendelian diseases will be found in healthy individuals [197]; and in many cases, may not actually be disease-causing mutations [18].

### 1.5.9 Data structures and computational efficiency

Most computational pipelines for genomic variant annotation and primary impact assessment are relatively efficient and can annotate variants obtained from large resequencing projects involving thousands of samples within a few minutes or hours even using a moderately powered laptop. This is typically achieved through two key optimizations: (i) creation of reference annotation databases and (ii) implementation of efficient search algorithms. Reference database creation refers to the process of creating and storing precomputed genomic data from the reference genome, which can be searched quickly to extract information relevant to each variant. This process needs to be performed only once per reference genome and most annotation tools have pre-computed databases for many organisms available for users to download.

Since these databases are typically quite large, efficient search algorithms are used together with appropriate data structures to optimize the search process. In ANNOVAR [238], each chromosome is subdivided in a set of intervals of size  $k$  and genomic features for a given chromosome are stored in a hash table of size  $L/k$ , where  $L$  is the length of the chromosome. Another approach, used by SnpEff, is to use an “interval forest”, which is a hash of interval trees [54] indexed by chromosome. Querying an interval tree requires  $O[\log(n) + m]$  time, where  $n$  is the number of features in the tree and  $m$  is the number of features in the result.

### 1.5.10 Conclusions

In Chapter 3 we introduce SnpEff [39] & SnpSift [40], two approaches we designed for efficiently performing functional annotations of sequencing variants. These packages allow annotating, prioritizing, filtering and manipulating variant annotations as well as combining several public or custom-created databases. It should be noted SnpEff was one of the first annotation packages and has become one of the most widely used annotation software in both research and clinical environments.

## 1.6 Genome wide association studies

A genome wide association study aims at identifying genetic variants associated to a particular phenotype. First, the genomes (or exome, depending on the study design) of affected individuals (cases) and healthy individuals (controls) need to be sequenced, variants called, and annotated. Then, the goal is to find variants that exhibit some statistical association with the trait or phenotype of interest, which could be a disease status (e.g. diabetic vs healthy), a biomedical measurement (e.g. cholesterol level), or any measurable characteristic (e.g. height). Since the genome is so large, patterns of mutations that suggest correlation may be encountered by chance, so we need to establish statistical significance in order to distinguish true associations from spurious ones. Like most studies, we will focus our discussion on SNVs, but most methods can be extended to other genomic variants.

### 1.6.1 Single variant tests and models

Consider a simple situation where there is only one variant in the whole genome for the cohort we are analysing. Since each individual has two sets of chromosomes, the variant can be present in one, both, or neither chromosomes, so the number of times a non-reference allele is present in an individual, is  $N_{nr} = \{0, 1, 2\}$ .

When the trait of interest is binary (e.g healthy vs disease), a cohort can be divided into cases and controls and we can build a 3 by 2 contingency table:

	Homozygous Reference ( $N_{nr} = 0$ )	Heterozygous ( $N_{nr} = 1$ )	Homozygous non-reference ( $N_{nr} = 2$ )
Cases	$N_{ca,ref}$	$N_{ca,het}$	$N_{ca,hom}$
Controls	$N_{co,ref}$	$N_{co,het}$	$N_{co,hom}$

Further assumptions about how many alleles are required to increase disease risk can

reduce this  $3 \times 2$  table to a  $2 \times 2$  table. In the “dominant model”, the effect of a mutated gene dominates over the healthy one, so one variant is enough to increase risk. The opposite, called “recessive model”, is when both chromosomes have to be mutated in order to increase risk [13, 44]. In these models, we can count how many cases and controls have at least one variant (dominant model) or two variants (recessive model). This simplifies the previous table, yielding a  $2 \times 2$  contingency table, than can be tested using either a  $\chi^2$  test or a Fisher exact test [13].

Two other commonly used models are the “multiplicative” and the “additive” models [13, 44]. In these models, a disease risk is assumed to be multiplied (or increased) by a factor  $\gamma$  with every variant present. In this case we cannot simplify the contingency table, so we assess significance using a Cochran-Armitage test [44].

### 1.6.2 Multiple variant tests

In a real case scenario there are thousands or millions of variants in a resequencing or genotyping study. We can extend the concept shown in the previous section by performing individual tests for each variant present in the cohort. Multiple testing can be addressed either by performing a correction, such as False Discovery Rate [13, 44], or using a stricter genome wide significance level. There are  $\sim 3 \times 10^9$  bases in the genome, but taking into account the correlation between nearby variants (linkage disequilibrium), the genome wide significance level is generally accepted to be  $p \leq 10^{-8}$ .

In order to check if the null hypothesis of a significance tests is adequate, a QQ-plot is used [44] (i.e. plotting the  $y = -\log(p)$  vs  $x = -\log[\text{rank}(p)/(N + 1)]$ , where  $N$  is the total number of variants). Adherence of the p-values to a  $y = x$  line on most of the range implies few systematic sources of association [13, 44]. If the p-values have a higher slope than the  $y = x$  line, there might be “inflation”, possibly due to co-factors, such as population structure (see section 1.6.4). If the inflation is not too high (e.g. less than 5%), this bias can be corrected by shifting the p-values towards the 45 degree line. More sophisticated methods

are explained in section 1.6.4.

### 1.6.3 Continuous traits and correcting for co-factors

The methods described so far are suitable for binary “traits” or “phenotypes”. Statistical methods that link genetic information to traits can also be used for continuous or “quantitative” traits (such as weight, height and measurements of cholesterol level). A linear regression can be used assuming the traits are approximately normally distributed [13, 44]. A significance test ( $p$ ) for linear models can be calculated using an  $F$  statistic, but more sophisticated methods are also available [13, 44].

Using linear models, it is easy to include known co-factors to correct for biases. For instance, if it is known that a phenotype increases with age or that males are more susceptible than females, age and sex can be added to the linear equation in order to correct for these effects [13, 44]. In a similar manner, we can add co-factors to binary traits using logistic regression.

### 1.6.4 Population structure

It is widely accepted that humans started in Africa and migrated to Europe, then to Asia and later to America [103]. Out of an initial population, a few individuals migrate and colonize a new territory. This implies that the genetic variety of the new colony is significantly reduced compared to the previous population, since the genetic pool is only a small “founder population”. The “Out of Africa” hypothesis implies that each new migration of humans from Africa to Europe produced a reduction in genetic variety, also known as a “population bottleneck” [103].

As we previously mentioned, each individual inherits two chromosome sets, a maternal and a paternal one. Through recombination a chromosome is formed by a crossover combining maternal and paternal chromosomes and then passed down, thus the offspring has two sets of chromosomes, one from each parent. This breaking and shuffling of chromosomes

every generation, increases genetic diversity. Nevertheless if variants are located nearby in the chromosome, the chances that they are separated by a recombination event are smaller than if they are further away from each other. This produces a correlation of close variants or “linkage disequilibrium” (LD). Nearby highly correlated variants are said to be in the same “LD-block” [103]. If a population has low genetic variety, the LD-blocks are large. So the African population has more diversity (smaller LD-blocks) and conversely, European, Asian and Amerindian populations have less diversity (larger LD-blocks) [103].

### 1.6.5 Population structure as confounding variable

Imagine that we have a cohort of individuals drawn from two populations ( $P_A$  and  $P_B$ ) and that individuals in  $P_A$  have much higher risk of diabetes than individuals from  $P_B$ . Now imagine that individuals from  $P_A$  have a variant  $v_A$  more often, but  $v_A$  is actually neutral and has no health effects whatsoever. If we do not take population factors into account our study would conclude that  $v_A$  is associated with increased susceptibility to diabetes, just because we see  $v_A$  more often in affected individuals. In this case it is clear that population structure is a confounding variable. We could avoid this problem by analysing each population separately [177], but this would cause a loss of statistical power since we would have fewer samples.

A population that results form inter-breeding of two or more previously separated populations is known as an “admixed population”. For instance the African-American population is a mixture of, roughly, 80% African and 20% European genomes [103, 13]. In the case that structure is confounding an analysis of an admixed population, such as an African-American cohort, it is not possible to perform a separate analysis of each sub-population [103].

The admixed population problem can be studied by performing a correction using the eigen-structure of the sample covariance matrix [177]. Samples can be arranged as a matrix  $C$  where each row is a sample and each column represents a position in the genome where there is a variant. The numbers  $C_{i,j}$  in the matrix indicate the number of non-reference alleles in a sample (row  $i$ ) at a genomic position (column  $j$ ). Since the allele can be present

in zero, one, or two chromosomes in each individual, the possible values for  $C_{i,j}$  are  $\{0, 1, 2\}$ . The covariance matrix is calculated as  $M = \hat{C}^T \cdot \hat{C}$ , where  $\hat{C}$  is the matrix  $C$  corrected to have zero mean columns. Usually, the first two to ten principal components of  $M$  are used as factors in linear models (see section 1.6.3) to correct for population structure [177].

Whether a cohort has any population structure and needs correction or not can be tested using two methods: a) plotting the projections of the first two principal components and empirically observing the number of clusters in the chart, or b) using a statistic of the eigenvalues of  $M$  based on Tracy-Widom's distribution [177].

### 1.6.6 Common and Rare variants

The “allele frequency” (AF) is defined as the frequency a variant appears in a population. Variants are usually categorized according to AF into three groups: Common variants ( $AF \geq 5\%$ ), “low frequency” ( $1\% < AF < 5\%$ ), and “rare variants” ( $AF < 1\%$ ). Common variants originated earlier in the population while rare variants are either relatively recent or selected against.

There are three main models for disease susceptibility [103, 90]: i) the Common-Disease-Common-Variant hypothesis (CDCV) assumes that if disease is common, it must be caused by a common variant; ii) the “infinitesimal hypothesis” proposes that there are many common variants each having a small effect on risk; and iii) the Common-Disease-Rare-Variant hypothesis proposes that there exists many rare variants, each one having large risk effects.

### 1.6.7 Rare variants test

The “rare variant model” assumes that multiple rare variants have large effects on a trait. The problem is that, since these variants are rare, huge sample sizes are required for tests to identify statistically significant associations. To overcome this problem, methods known as “burden tests” collapse groups of rare variants that are hypothesised to have similar effect on gene or protein activity and perform statistical significance tests on the group [141]. An

example of collapsing technique is to count the number of rare variants in the genomic region surrounding an exon or a gene and apply a Fisher exact test, as shown in section 1.6.1. A limitation of some burden tests is that they implicitly assume that all rare variants have the same direction of effect, although in reality they might have no effect, be deleterious, or protective [141, 251].

Several techniques that allow weighting rare variants by collapsing them using a kernel matrix. This allows to incorporate other information, such as allele frequency and functional annotations. It can be shown that the statistic induced by kernel weighting functions follows a mixture of  $\chi^2$  distributions and there is an efficient way to approximate it [141, 251], avoiding computationally expensive permutation tests.

## 1.7 Epistasis

William Bateson first described epistasis in 1907 [233] assessing a discrepancy between the predicted segregation ratios and the observed ones [182]. The term epistasis, which literally means “standing upon”, was used to describe “characters” layered on top of other each other thus masking their expression. This original definition describing the situation in which the actions of one locus mask the allelic effects of another locus is an extension of dominance where a completely dominant alleles mask the effects of the recessive allele at the same locus [31, 52].

Nowadays the term epistasis is not only used to describe the original definition [52], but also often interpreted as mutations in two genes producing a phenotype that is surprising considering the individual effect of each mutation. Furthermore, in some contexts epistasis is used to refer to a broad range of gene-gene interactions, many complex interactions among genetic loci or even interaction between genes and the environment [182]. Three categories of epistasis commonly used by geneticists are [182, 258]:

- Functional epistasis: The molecular interactions between proteins, usually consisting

of proteins within the same pathway or within a complex.

- Compositional epistasis: Describes the traditional usage of epistasis as described by Bateson (i.e. masking of one allelic effect by an allele at another locus).
- Statistical epistasis: This terminology is attributed to Fisher and defined as a deviance from additive genetic effects.

These concepts imply that analysis of epistasis can be used to infer functional relationships between genes [154], genetic pathways' structure and function as well as evolutionary dynamics [182]. Some authors even relate the analysis of epistatic gene interactions patterns to the fundamentals of systems biology [182].

Epistasis can be classified by the way a deviation of a double-mutant (having one mutation at each loci) organism's phenotype differed from the expected neutral (non-interacting) phenotype [154]. A typical example is a mutation in one gene impairing a whole pathway, thus masking the consequence of mutations in other genes of the same pathway [154]. An interaction is known as "synergistic" or "synthetic" when the double mutant has a more extreme phenotype than expected based on the phenotype of the two individual mutants. When the phenotype is less severe than expected, then there is a "alleviating", "diminishing returns" or "antagonistic" interaction, which is often attributed to gene products operating in series within the pathway.

Often, a phenotype in human genetics is qualitative and dichotomous, for instance indicating presence or absence of disease. [52]. Thus mathematical models calculating the joint action of more than one loci focus on the penetrance (the probability of developing disease given genotype). Assuming an allele is required at both loci in order to express the trait, the effect of allele  $A$  can only be observed when allele  $B$  is also present. This means that the effect at locus  $A$  appears masked by locus  $B$  and vice-versa [52], which is not precisely analogous to what Bateson described since in Bateson's definition if factor  $B$  is epistatic to factor  $A$ , then factor  $A$  is not expected to be epistatic to factor  $B$  as well [52].

A mathematical definition of epistatic interaction as “departure from neutrality” requires defining neutrality and measuring phenotype.

- *Phenotype* is often measured using the concept of fitness, particularly in many large-scale genetic interaction studies, since it is relatively easy to measure by population allele frequencies or growth rates of microbial cultures [154]. Different measures of fitness can be used in epistasis: i) exponential growth rate of mutant strain respect to wild type ; ii) the increase in population in one wild-type generation; and iii) the relative number of progeny (in one wild-type generation) [154]. Based on this, four mathematically different definitions of interaction have been used (namely *product*, *additive*, *log*, and *minimum*) [154], but even though some definitions yield identical results under some conditions, an alternative definition choice can lead to different consequences[154].
- *Neutrality* predicts the phenotype of an organism without interacting mutations. Genetic interaction studies have differed in their choice of neutrality models, generally using either a multiplicative or a minimum mathematical function. A multiplicative model predicts fitness to be the product of the corresponding single-mutant fitness values. The minimum model is simply the minimum neutrality of the expected results form non-interacting mutations (e..g the fitness of the less-fit mutant). All the above examples of fitness measures yield the same set of genetic interactions under this neutrality definition. For example if each mutation disrupts a distinct pathway limiting cell growth in a way that one mutation is substantially more limiting than the other, the double mutant might is expected have same result as the most-limiting single mutant [154].

It has been shown that the choice of definition can dramatically alter the resulting set of detected interactions [154]. To evaluate this Mani et al. [154] applied all four definitions of interactions to two experiments providing quantitative growth-rate measurements of cell

populations. They show that: i) the *additive* and *log* definitions have different biases; ii) the *product* and *log* definitions are equivalent for deleterious mutations; iii) the *product* definition can reveal functional relationships missed by the *minimum* definition; and iv) interaction networks inferred based on the *minimum* and *product* definitions differ greatly. This leads to the question on which definition to use. By examining the deviation distribution of expected (double-mutant) phenotype from the observed phenotype they found that *product* and *log* definitions not only are the closest to the ideal, but also are practically equivalent when single mutants are deleterious.

The presence or absence of a trait are extreme aspects of “perturbation in a complex system”, but there are no reasons to expect all forms of epistasis to follow this pattern [182]. When applied to quantitative traits, epistasis also describes a situation in which the phenotype cannot be predicted by the sum of the phenotypes of its single-locus component [31]. Many epistatic QTL interactions have been detected in model organisms leading to the conclusion that epistasis makes a large contribution to the genetic regulation of complex traits [31].

### 1.7.1 Epistasis is ubiquitous

One of the most common definition of epistasis is departure from additive effects. Nevertheless, there is no reason to think that traits should be additive based on a purely biological perspective [261] since biology is riddled with non-linearity such as genetic networks which exhibit binary states, ligand - receptors concentration having sigmoid-like responses, concentration saturations of substrate - enzymes reactions, sharp transitions created by cooperative protein binding, the pathways constrained by rate-limiting inputs, etc. [261]. It has been asserted that epistatic effects are not isolated events, but ubiquitous [233] and probably inherent properties of biomolecular networks. The thought that epistasis in the classical sense may be ubiquitous has been partially confirmed from mutational studies [182]. Genetic studies of synthetic traits, which occur only when multiple loci or pathways are all disrupted

in model organisms, have identified instances of interacting genes revealing that epistasis may be pervasive [261]. Researchers [182] looking for interactions induced by systematically over-expressing genes in *Saccharomyces cerevisiae*, found that about 15% of studied genes induced growth defects with most over-expression not matching the phenotypes of individual deletions.

### 1.7.2 Epistasis examples

**Non-human** Several genotype-phenotype patterns are known to be caused by epistasis in animal and model organisms. Classic examples including [31]:

- Coat colour in mammals has been one of the most common examples. In pig, the dominant allele at the KIT locus confers white color coat and is dominant over all locus conferring darker color (melanocortin 1 receptor or MC1R). This can be determined in individuals with the recessive KIT genotype showing what was classically termed ‘dominant epistasis’, yielding a non-Mendelian segregation ratio of 9:4:3 (instead of 9:3:3:1) [31, 182].
- *Drosophila* provides another classic example with eye color determination. *Drosophila* eye pigmentation (scarlet, brown, or white) is determined by the synthesis of two drosopterins: brown pigments (from tryptophan) and red pigments (from GTP) [233]. A mutation that prevents production of the brown pigment results in a fly with red eyes and a mutation preventing red pigment results in a fly with brown eyes. Flies with a mutation in the white gene, synthesize neither red nor brown pigment, resulting in a fly with white eyes regardless of the genotype at the brown or scarlet loci [233].
- Dozens of quantitative traits indicating strong epistasis in mouse and rat [213] have been identified in a panel of chromosome substitution strains. The effects attributed to the strain-specific region of donor chromosomes exceeds by a median eightfold the expected effect of the donor genome.

- Genetic interaction have been studied in a systematic and large-scale manner in *Saccharomyces cerevisiae* [118, 228]. Analysis of quantitative traits loci (QTL) for transcripts levels in a two strain cross demonstrated epistatic interaction for 67% of studied pairs (first the strongest QTL was found and then the strongest remaining QTL conditional on the first genotype was selected) [25].
- In a study comparing three *Drosophila* inbred lines (*Drosophila melanogaster* Genetic Reference Panel) and a large out-bred and inter-cross derived population [111], a set of candidate SNPs was selected by assessing allele frequency changes between the extremes of the distribution for each trait. The researchers found that the majority of these SNPs participated in at least one epistatic interaction [111]. Using this information from epistatic interacting loci they were able to infer networks affecting quantitative traits [111].

**Human** Few instances of epistasis in common human diseases have been discovered and well-replicated so far, despite considerable efforts [261]. Although many instances of epistasis related to human disease have been published, with examples from type 2 diabetes [248], bipolar effective disorder [117], coronary artery disease [230], and autism [56]; some authors suspect these might be statistical features in the association studies because only a few have functional basis [182]. Perhaps the best examples of epistatic interactions in humans include:

- Interactions involving at least one locus with a large effect such as HLA [261]. Two different interactions involving HLA alleles and ERAP have been discovered in GWAS from ankylosing spondylitis and psoriasis where the HLA alleles have odds ratios of 40.8 and 4.66 respectively [77, 172]. In the autoimmune disease multiple sclerosis researchers found evidence of genetic interactions between two histocompatibility loci known to be associated with the disease (HLA-DRB5\*0101 in DR2a and HLA-DRB1\*1501 in DR2b) [97]. In Type 1 diabetes HLA is assumed to act non-additively with all other risk alleles (HLA has have an effect of 5.5) [16].

- In Hirschsprung's disease an interaction between RET and EDNRB was uncovered by a genome-wide linkage study (RET having a log-odds of 5.6) [32].
- The ACE gene (angiotensin I converting enzyme) has an epistatic interaction with AGTR1 gene (angiotensin II type 1 receptor ) gene, significantly increasing risk of myocardial infarction when the “D-allele” in ACE is present in patients carrying a particular AGTR1 allele [227].
- Two different sets of interactions are assumed to be responsible for variation in triglyceride levels. Notably, the interactions depend on the patient's sex: in females the interaction involves ApoB and ApoE; and in males the interaction involves the ApoAI/CI-II/AIV complex and low-density lipoprotein receptor LDLR [169].
- Sickle-cell anemia is regarded as a Mendelian trait but is modified by epistatic interactions as evidenced by the fact that patients homozygous for two polymorphisms near the *Gγ* locus have only mild clinical symptoms [171].
- Elevated blood serum cholesterol levels in humans is associated with an ApoE allele depending on the genotype at the LDLR (low density lipoprotein receptor) gene locus [180].

### 1.7.3 Epistasis and evolution

From an evolutionary perspective, some authors argue that the non-linear nature of epistatic interactions between polymorphic loci is the genetic basis of canalization (the robustness or ability of a population to produce the same phenotype regardless of environmental variability) and speciation [111].

It has also been pointed out that interactions have an important influence on evolutionary phenomena such as genetic divergence and affects the evolution of the structure of genetic systems [182] based on studies and models showing that epistasis can have a limiting role on the possible paths that evolution can take [163].

Theoretical arguments that date back to Fisher assert that when genes interact there is evolutionary pressure to promote their genetic linkage as a means of enhancing the co-inheritance of favourable allelic combinations [82]. Under this assumption linkage can facilitate the maintenance of epistatic interactions and vice versa, thus explaining some molecular evolution complexity [182]. This has been supported by analysis of complex gene regulation patterns in localized genomic regions [21]. For variety of organisms (such as yeast, *Caenorhabditis*, *Drosophila*, higher plants, and mammals) genes sharing expression patterns are more likely to be in proximity [113]. This evidence shows that regional controls of chromatin structure and expression may give rise to gene clusters by promoting their coregulation [181].

#### 1.7.4 Missing heritability

At the dawn of the “GWAS era” in 2002 it was hypothesised that there existed a large class of genetic traits for which GWAS would fail, namely purely epistatic models containing no additive or dominance variation at any of the susceptibility loci. Thus association case/control methods “will have no power if the loci are examined individually” [60]. Furthermore, it was mathematically shown that for such models maximizing the broad sense heritability (under some constraints) is equivalent maximizing gene interaction variance [60].

In a seminal series of papers [261, 262] further mathematical proof of the link between epistasis and heritability was provided. The authors claim that missing heritability arises by an overestimation of the denominator that happens when epistasis is ignored [261]. This overestimation, called “phantom heritability”, was shown to inflate the denominator over 60% in Cohn’s disease, thus accounting for up to 80% of the missing heritability [261]. Even though the prevailing view among geneticists is that interactions play at most a minor role in explaining missing heritability, their work shows that simple and plausible models can give rise to substantial phantom heritability [261]

In moderately heritable complex diseases for which single-locus GWAS analyses have not

accounted for the predicted phenotypic variance these epistatic models provide one possible explanation so it is worth pursuing a hypothesis of interacting loci [60].

### 1.7.5 Detecting epistatic interactions

Linkage disequilibrium (LD) between close sites is the result of un-recombined chromosome blocks within common ancestry [195]. However LD between widely separated sites suggests epistatic selection forces are at work [82, 127]. In an analysis using the Yorubian population (from Ibadan, Nigeria) of the HapMap dataset, patterns of LD were quantified and the significance of overall disequilibrium per chromosome was evaluated of using randomization [127], showing an excess of long range associations on all 22 autosomes. Although this is suggestive of epistasis, other hypotheses should not be ruled out: i) population admixture has been proposed to explain unusual patterns of long range LD [186]; ii) recombination between distant chromosome blocks may not completely erase LD caused by drift even in a population at demographic equilibrium; iii) bottlenecks are particularly effective at generating long-range LD; iv) hitchhiking of linked sites with a positively-selected mutation can generate large haplotype blocks; and v) large inversion and other structural variation alter recombination patterns thus causing LD over unusually large regions [15].

Under the assumption that long range LD can hint at epistasis due to physical protein interactions, Wang et al. created LDGIdb [239], a catalogue of over 600,000 pairs of SNPs showing strong long-range linkage disequilibrium, i.e. pairs of SNP pairs that were either located on different chromosomes or on different LD blocks and had  $r^2 \geq 0.8$  [239]. However such a simple approach may be of little utility because of technical issues that must be taken into account when performing such association tests: i) commonly used measures of LD (such as  $r^2$  and  $D'$ ) are known to give rise to large linkage for variants with minor allele frequencies (MAF) near 0 [127]; and ii)  $r^2$  is not corrected for multiple testing. A better alternative is to measure the probability that a large value of the disequilibrium  $D$  is observed if there is no association. The aforementioned problems can be corrected by conditioning the probability

on the sampled allele frequencies at the two loci. This method has the analytical advantage that the probability asymptotically converges to a Fisher's exact test [127].

Another approach is to implicitly test over and under-representation of allele pairs in a given population, i.e. to analyse imbalanced allele pair frequencies [2]. The underlying theory is that such allele pairs are under Dobzhansky-Muller incompatibilities which establishes a fitness bias favouring individuals that inherit over-represented allele combinations [2]. Based on this, Ackerman et al. [2] studied a population of 2,002 mice in family trios. They performed a  $\chi^2$  test correcting by confounding factors (such as allele frequencies, family structure and allelic drift) based on inspecting  $3 \times 3$  contingency tables of all possible two-locus allele combinations. They claim that their methodology can detect more interactions than using independent markers and as a result they were able to identify 168 LD block pairs with imbalanced alleles [2].

By exploiting the intense selective pressures imposed by the process of inbred mouse populations, it can be expected that clusters of functionally related genes are likely to be selected for co-adapted allelic combinations in genes that influence fitness and survival. This hypothesis would result in regions of linkage disequilibrium (LD) among inbred strain genomes that should occur more often than expected by chance [181]. In a study using 60 inbred mouse strains [181], the authors study LD using permutation tests and show that extreme patterns of LD give rise to a scale-free network architecture. Further pathway analysis identifies biological functions underlying several of these networks, hinting that selective factors acting to generate LD networks during inbreeding reflect functional interaction [181].

In the next sub-section we introduce methods combining GWAS and epistatic analysis to find epistatic loci affecting disease risk.

### 1.7.6 Epistasis & GWAS

In recent years there have been a growing number of GWAS. Most of them have used a single-locus analysis strategy, in which each variant is tested individually for association with a

specific phenotype [53]. Some researchers mentioned that it may be inadequate to describe relationships between genotype and phenotype in complex disease by simply summing the modest effects from several contributing loci [60]. Nevertheless, the extent to which epistasis is involved in complex traits is not known so we cannot assume that epistasis will be found for every trait in every population [31]. However epistasis has been overlooked and should to be routinely explored in complex trait studies [31]. This is particularly important for researchers of moderately heritable complex diseases for which locus-by-locus analyses have not accounted for the predicted genetic variance. In this case there could be value in pursuing a hypothesis of epistatic loci [60] that owing to their interaction, might not be identified by using standard single-locus tests [53]. It is also hoped that detecting such interactions will allow elucidation of biological pathways that underpin complex disease [53].

Recent GWAS studies explored genome-level identification of epistatic interactions [2]; and even though methodological and sample size progress has been made, these could hardly identify a significant number of interactions. However failure to detect epistasis does not rule out its presence [261]. In theory a sufficient number of contributing purely epistatic interactions could account for all the variation in disease status for any prevalence [60]. Nevertheless, when the genetic model of disease is purely epistatic (i.e. no additive or dominance at any of the susceptibility loci), then association methods analysing a single locus at a time cannot detect the loci [60]. Furthermore there could be an  $n$ -way purely epistatic model for which no joint analysis of two, three, or  $n - 1$  loci shows any evidence of association. This leads to the concern that even assessment of all “ $(n - 1)$  way” interactions among candidate loci may not be sufficient for detection of the contributing loci [60].

Another reason why complex human phenotypes fail to find evidence for epistatic interactions may simply be that analytic methods inherently exclude epistasis [60]. For example individual interaction effects are expected to be much smaller than linear effects, and the sample size required to detect a variant scales inversely with the square of the effect size. The main obstacle is attributed to the exponentially large number of statistical hypotheses

tested when comparing all markers against all other markers in a genome analysis [2]. As an example provided by Zuk et al. [261], consider two variants with frequency 20% and increasing risk by 1.3 fold, which is a large effect. In such a case, assuming 50% power, a significance level of  $5 \times 10^{-8}$  and equal number of cases and controls, the sample size required for single loci analysis would be 4,900. In comparison, the sample size required to detect pairwise interaction between those two variants using the same power and an appropriately corrected significance level is roughly 450,000, so a researcher studying 100,000 samples would discover all single acting loci but would find little evidence of epistatic interactions, which may be the reason why geneticists that have tested for pairwise epistasis have found few significant signals [261]. It should be noted that even though GWAS involving over 500,000 samples are not available at the moment, studies using sample sizes in this order are expected to become available within the next couple of years.

Existing approaches for identifying interactions in the context of GWAS can be grouped into five broad categories [147]:

1. **Exhaustive search** methods extend classical single-locus GWAS statistics such as the Pearson's  $\chi^2$  test or logistic regression. For instance, using the definition of epistasis as "departure from a linear model" [53], in a logistic regression model the input for sample  $s$  analysing loci  $i$  and  $j$  would include terms with each of the genotypes ( $g_{s,i}$  and  $g_{s,j}$ ), as well as an "interaction term" ( $g_{s,i} \cdot g_{s,j}$ ) [52].

$$\begin{aligned} P(d_s | g_{s,i}, g_{s,j}) = & \phi[\theta_0 + \theta_1 g_{s,i} + \theta_2 g_{s,j} + \theta_3(g_{s,i}g_{s,j}) \\ & \dots + \theta_4 c_{s,1} + \dots + \theta_m c_{s,N_{cov}}] \end{aligned}$$

where  $d_s$  is disease status,  $\phi(\cdot)$  is the sigmoid function,  $c_{s,1}, c_{s,2}, \dots$  are covariates for sample  $s$ . Logistic models involving interactions between more than two variants can be defined similarly, but require more parameters and extremely large samples are

required to accurately fit them.

It should be noted that the number of tests necessary to evaluate all two-, three- and four-way interactions for 30-60 candidate loci, has a range similar to the number of tests suggested for a single GWAS, thus searching for n-way interactions among all the markers would be impracticable [60]. Other approaches [169, 59, 198, 51, 259] although promising, most have only been applied on small data sets [257]. Methods based on brute-force searches such as combinatorial partitioning [169], and multifactor-dimensionality reduction [198] are impractical for large data sets [257]. Nevertheless it was shown [147] that it can be feasible to perform GWAS level analysis for two interacting sites. Simple methods which explicitly consider interaction pairs can actually achieve reasonably high power with realistic sample sizes under different interaction models with some marginal effects, even after adjustments of multiple testing using the Bonferroni correction.

Exhaustive search methods exists for identifying epistatic variants affecting continuous phenotypes and quantitative trait loci (QTL). In this case, matrix algorithm optimizations can significantly speed up computations. For instance FastEpistasis applies an efficiently parallelized QR decomposition to derive least squares estimates of the interaction coefficient and its standard error [212]. This allows it to handle all pairs of 500,000 variant in a population of 5,000 individuals in roughly one CPU year, which can be run in a little bit more than a day on a 256 CPU cluster [212].

2. **Linkage disequilibrium** methods use patterns in disease population under two-locus disease models [258]. Association can be estimated assuming that deviation of the penetrance from independence at an individual locus creates linkage disequilibrium (LD) even if two loci are unlinked [258]. In Zhao et al. [258] the authors, based on the assumption that two disease-susceptibility loci are in Hardy-Weinberg equilibrium (HWE), show that in the presence of interaction the two loci will be in linkage

disequilibrium in the disease population. They develop a test statistic to detect of deviations from LD by comparing the difference in the LD levels between two unlinked loci between cases and controls. Under the null hypothesis, this test statistic asymptotically converges to a central  $\chi^2$  distribution. Their power simulations suggest that in general this LD-based test statistic has much smaller p-values than those of logistic regression analysis concluding that their test has higher power than logistic regression. Nevertheless, their model does not account for cofactors, thus making it unsuitable in multi-ethnic GWAS where population stratification may confound disease risk.

3. **Stochastic search** methods use sampling to infer whether a locus is an individual risk locus, epistatically affects disease risk, or has no effect (i.e. background locus). A Bayesian approach for genome-wide case-control studies denoted ‘bayesian epistasis association mapping’ (BEAM) [257] is a representative example of this type of method. BEAM treats the disease-associated markers and their interactions using a Bayesian partitioning model and computes the posterior probability using Markov chain Monte Carlo. The method uses predictors in the form of genetic marker loci divided into three groups: i) markers not associated with disease, ii) markers individually contributing to disease risk, and iii) markers that interact with each other [257]. Membership of each marker in each of the three groups is defined by the prior (Dirichlet) distributions. Given a prior distributions for regression coefficients values given by group membership, a posterior distribution can be generated using MCMC simulation [53]. At the end, it uses a statistic (called B-Statistic) to infer significance from the samples in MCMC. Although it avoids explicitly computing all interactions the method could in theory find high-order interactions. Since BEAM was originally designed for genotyped markers, its power can be hampered by allele frequency discrepancies between unobserved disease loci and linked genotyped markers. This is a common problem when using indirect markers and the authors show that in an extreme case when the MAF discrepancy was maximized all tested methods had little power to detect interaction

associations. In the original paper, the authors apply BEAM to a data set containing 116,204 SNPs genotyped for 96 affected individuals and 50 controls for an association study of age-related macular degeneration (AMD). Unfortunately BEAM did not find any significant interactions, most likely due to the small sample size. Runtime and power are primarily determined by the number of MCMC rounds with a suggested number of MCMC iterations as the quadratic of the number of SNPs. This is a main factor limiting applicability of the algorithm [147], so BEAM cannot easily be applied to large GWAS studies because computational limitations make it unsuitable to handle over 500,000 markers with sample sizes of 5,000 or more individuals, which are now commonly sequenced or genotyped [53].

4. **Conditional search** methods usually perform analyses in stages [147]. A small subset of significant loci is identified in the first stage, typically using single locus association statistics. Then this subset is mined using multi-locus association using an exhaustive method. A well known approach in this category is “stepwise logistic regression” which works as follows: i) all markers are individually tested for association with disease using a logistic regression model; ii) loci are ranked based on the results of single-locus tests; iii) the top (usually 10%) are selected for epistatic association, and iv) all two-way (or three-way) interactions are tested. Even this stepwise approach can become computationally intractable for high-order interactions [257]. Analysis of stepwise logistic regression approach to identify two-way and three-way interactions demonstrated that searching for interactions in genome-wide association mapping can be more fruitful than traditional approaches that exclusively focus on marginal effects [257]. As a counter argument for stepwise logistic regression, we should take into account that the effect of one locus is altered or masked by another locus (in the presence of epistasis), thus power to detect the first locus is likely to be reduced and the joint effects will be hindered by their interaction [52]. Methods based on conditional search can greatly reduce the computational burden by a couple of orders of magnitude,

but with the risk of missing markers with small marginal effect [147].

5. **Machine learning** approaches can also be used to detect epistasis. A popular approach uses Random Forests [147] or other regression tree partitioning approaches based on classification. In this context, trees are constructed using rules based on the values of a predictor variable such as a SNP to differentiate observations such as case-control status [53]. A popular rule selection mechanism is to use the variable that maximizes the reduction in Gini impurity [132] at each node (intuitively, when child nodes have lower impurity from a split based on an attribute each child node will have purer classification). Random Forests are constructed by drawing samples with replacement from the original sample. A classification tree is created for each bootstrap sample, but only a random subset of the possible predictor variables is considered. This results in a ‘forest’ of trees have been trained on a particular sample of observations. [53]. Instead of trying to create a monolithic learner, this type of methods called “ensemble systems” attempts to create many heterogeneous “weak” (or simple) learners. The outcomes of these heterogeneous systems are combined to create an improved model [147].

In Li et al. [147], the authors create an extension of the AdaBoost algorithm where they incorporate an importance score based on Gini impurity to select candidate SNP in a way that genotype frequencies from the two classes (case and control) are expected to be more different. Decision trees are usually built with binary splits, but since genotype data takes three possible values  $\{0, 1, 2\}$ , they also extended their method to create a ternary split. AdaBoost draws bootstrap samples to increase the power of a weak learner by weighting the individuals when bootstrapping. So when a weak learner misclassifies an individual, the weight of that individual is increased, and hard to classify individuals are more likely to be included in future bootstrap samples. The ensemble classifier votes by weighting weak learner instances by training set accuracy. [147]. Using simulation, they claim that their method outperforms similar ensemble

approaches, as well as statistical methods (logistic regression), although they mention performance degradation when the risk allele frequency is low [147].

Although all these models have advantages under some assumptions, none of them seems to be a “clear winner” over the rest [53]. All of these models suffer from the increase in number of tests that need to be performed, which raises two issues: i) multiple testing, and ii) computational feasibility. So far, no method for epistatic GWAS has been widely adopted and there is need of different approaches to be explored. In Chapter 4 we propose an approach to combine co-evolutionary models and GWAS epistasis of pairs of putatively interacting loci.

### 1.7.7 Conclusions

Genome wide association studies have traditionally focused on single variants or nearby groups of variants. An often cited reason for the lack of discovery of high impact genetic risk factors in complex disease is that these models ignore interactions among loci [53] which has recently been pointed out as a potential cause of the “missing heritability” problem [261, 262]. With interactions being so ubiquitous in cell function, one may wonder why they have been so neglected by GWAS. There are several reasons: i) models using interactions are much more complex and by definition non-linear [86], ii) information on which proteins interacts with which other proteins is incomplete [234], iii) in the cases where there protein-protein interaction information is available, precise interacting sites are rarely known [234], and iv) protein interactions are not the only sources of epistatic loci, other types of interaction loci are less known and may be even harder to map. Due to the lack of knowledge about interaction loci, we need to explore all possible combinations, thus the number of  $N$  order interactions grows as  $O(M^N)$  where  $M$  is the number of variants [67]. This requires exponentially more computational power than single locus models. This also severely reduces statistical power, which translates into requiring larger cohorts, thus increasing sample collection and sequencing costs [67].

In Chapter 4 we develop a computationally tractable model for analysing putative interaction of pairs of variants from GWAS involving large case-control cohorts of complex disease. Our model is based on analysing cross-species multiple sequence alignments using a co-evolutionary model in order to obtain informative interaction prior probabilities that can be combined to perform GWAS analysis of pairs of non-synonymous variants that may interact.

## 1.8 Coevolution

In a book published in 1859 entitled “*On the origin of species by means of natural selection*” [62], Charles Darwin introduced the concept of co-evolution referring to the coordinated changes occurring in pairs of organisms. In another of his books “*On the various contrivances by which British and foreign orchids are fertilised by insects*”, first published in 1862 [63]; Darwin further explored this concept and providing more detailed examples. By observing the relationship between the size of orchids’ corolla and the length of the proboscis of pollinators, Darwin predicted the existence of a new species able to suck from a large spur which was later confirmed [67].

Coevolution originally referred to the coordinated changes occurring in pairs of organisms to improve or refine interactions. This concept was later extended to pairs of proteins or more generically, any pair of biomolecules which can be within the same organism [67]. The modern use of co-evolution methods in genetics is often attributed to Dobzhansky’s [71] and Elrich’s [76] seminal works that were published in 1950 and 1964 respectively. In recent years, much effort has been dedicated to research of coordinated sequence changes in proteins (and genes) where coevolution could be an important and widespread catalyst of fitness optimization [67].

Distinct allele combinations in co-evolving genes interact to confer different degrees of fitness. If this fitness difference is large, selection for alleles could maintain allelic association

even between unlinked loci [199], thus co-evolving genes are expected to maintain their interaction by pressures favouring compensatory mutations [199]. Under this hypothesis, genetic loci may be invariable due to their functional or structural constraints but these constraints may change subject to mutations in their functional counterpart [79]. In many cases, selective advantages for a specific allele pair could fixate the optimal allele pair in the population [199].

**Co-evolution examples** In the absence of a clear positive control, identifying gene pairs that is certainly co-evolving are a difficult task [199]. Here, some well known examples of co-evolution in humans are introduced:

- HLA ligand and killer-cell immunoglobulin-like receptor (KIR) are two genes located on different chromosomes forming a well established interacting immune-response pair. Their allele frequencies are highly correlated in human populations as one expects under allele matching selection [218].
- A remarkable similarity in the phylogenetic trees of ligands (such as insulin and interleukins) and their corresponding receptors was observed. This coevolution is proposed to be required for maintaining their specific interactions [179].
- Researchers found that ligands and their G-protein coupled receptors have co-evolved so that each subgroup of ligands has a matching subgroup of receptors [94].
- In Hsp90 and GroEL heat-shock proteins, co-evolution was detected in “almost all” functionally or structurally important sites [79].
- GroESL is involved in the folding of a wide variety of other proteins with the folding activity mediated by the co-chaperonin GroES [202]. It was recently shown that different overlapping sets of amino acids co-evolve between GroEL and GroES [202].

- Gamete recognition genes ZP3 and ZP19 are highly polymorphic among humans and located on different chromosomes. Putative interaction between these genes was recently inferred [199].
- *Helicobacter pylori* is the main cause of gastric cancer. Host-pathogen interaction accounted for most of the difference in the severity of gastric lesions in the populations analysed. For instance African *H. pylori* ancestry was relatively benign in population of African ancestry but was deleterious in individuals with substantial Amerindian ancestry [128]. This is an example of co-evolution modulating disease risk.

### 1.8.1 Basic co-evolution inference models

In this section we review the first methods aimed to uncover co-evolution. These “basic methods” serve not only to understand the historical perspective but also they are the basis of more advanced methodologies described in section 1.8.2.

**Phylogenetic tree similarity** Proteins and their interaction partners co-evolve so that divergent changes in one are complemented by their interaction partner. These changes can be manifested by “similar evolutionary trees” [94]. Thus phylogenetic similarity approaches can successfully be applied for protein-protein coevolution assumed to be caused by physical interactions. These kind of methods have been shown to be capable of identifying interaction partners, such as ligand-receptor pairs [67].

Similarly, evolutionary relationships within protein families can be mined to predict physical interaction specificities [191]. Duplicate genes (paralogs) can diverge in a way such that new binding specificities develop, thus the underlying hypothesis is that interacting proteins exhibit coordinated evolution and tend to have similar phylogenetic trees. This was first demonstrated in a study of chemokines and their receptors showing phylogenetic tree similarities [94].

**Correlated mutations** Although some methods based on phylogenetic tree similarity exists, the majority of co-evolutionary methods focuses on analysis of multiple sequence alignment [199]. Proteins have evolved to interact or function in specific molecular complexes and the specificity of these interactions is essential for their function. Consequently, residue contacts constrain the protein sequences to some extent [178]. In other words, it is reasonable to assume that evolution of sequence changes on one of the interacting proteins must be compensated by mutations in the other [178]. It should be noted that this relationship between co-evolution and interaction is not symmetrical since co-evolution does not imply physical interaction [79]. This is emphasized by the fact that co-evolution between clusters of sites not in contact has also been shown [187].

It has long been suggested that correlations in amino acid changes can be used to infer protein contact, thus helping predict tertiary protein structure [83, 164, 30, 67]. A large number of genomes and protein sequences have become available in recent years enabling the analysis of co-evolution by means of statistical inference of covariation patterns based on multiple protein sequence alignments [30, 30], which has been a fruitful technique for predicting contacting residues in the structure. This interdependent changes in amino acids was formulated for the first time by the “covarion model” [83] and applied in multiple sequence alignments of a family of homologue proteins [67]. Statistical methods to find correlated mutations between pairs of proteins can identify putative interaction sites in protein pairs [67], but we should keep in mind that correlated mutations suggesting compensatory changes between residues can be due to several factors different than direct contact, such as physical proximity, catalytic action, binding sites, or even maintaining folding stability.

One of the first attempts of statistical inference of co-evolving loci pairs was performed by Gobel et. al in 1994. In their seminal paper they point out that *“maintenance of protein function and structure constrains the evolution of amino acid sequences... [sequence alignments] can be exploited to interpret correlated mutations observed in a sequence family as an indication of probable physical contact in three dimensions”* [92]. They analysed correlations

between different positions in a multiple sequence alignment and used such correlations to predict contact maps. In their study of 11 protein families they compare their results with experimentally validated contact maps determined by crystallography, showing prediction accuracy up to 68%.

The promise of developing methods for predicting amino acid contacting pairs from sequence information alone was radically different from and more applicable than traditional docking methods [178]. This lead to the development of methods for detecting correlated changes in multiple sequence alignments with the primary objective of using them to detect protein interfaces in interacting molecules [178], thus facilitating protein structure prediction. It was demonstrated that the correlated sequence information was enough to select the right inter-domain docking solution amongst many alternatives.

Correlation and mutual information (MI) have been used to assess co-evolution but they do not take the evolutionary interdependence between protein residues into account [79]. Phylogenetic relationships can inflate these co-evolutionary measures, thus one of main limitations of these methods has been their inability to separate phylogenetic linkage from functional and structural co-evolution [79]. Some methods partially correct these effects but while some studies [91] claim that these would require alignments of at least 125 sequences, while other studies [164] suggest that they may require in the order of 1,000.

**Phylogenetic correction** Mutual information (MI) measures the reduction of uncertainty about one position given information about the other. When used as a measurement for co-evolution, MI can be confounded by several factors such as structural and functional constraints, and the background sum of contributions from random noise and shared ancestry. In an attempt to improve MI's signal to noise ratio by eliminating or minimizing the second factor, a model postulated by Dunn et al. [73] tries to factorize these terms in order to estimate a correction. They propose that each amino acid position in the MSA has a propensity toward the background MI (related to its entropy and phylogenetic history) and estimate the

joint background MI as the product of their propensities. It follows that a joint background correction term can be approximated as product of the average background MI divided by the average overall MI of all positions in the MSA, which they call the average product correction (APC) [73]. They show that APC is a metric than can accurately estimate MI in the absence of structural or functional relationships (i.e. the null model) [73]. Finally, by assuming the null model to be normally distributed, a p-value can be inferred using a Z-score.

Another method, CAPS [79], compares transition probability scores from the blocks substitution matrix (BLOSUM) between two sequences at the sites being analysed for interaction. An alignment-specific BLOSUM matrix is applied depending on the average sequence identity. Co-evolution between protein sites is estimated by the correlation in the pairwise variability with respect to the mean pairwise variability per site [79]. A limitation of this method arises when sequences are too divergent, since an alignment including highly divergent sequence groups could show unrealistic level of pairwise identity (BLOSUM values are normalized by the time of divergence between sequences to reduce the impact of this). Another problem common to many MSA-based co-evolutionary methods is that constant amino acid sites, which are very likely to be functionally important, cannot be tested for [79].

**Evolutionary timespan** What is the appropriate evolutionary time scale required in a multiple sequence alignment in order to perform a co-evolutionary analysis? Co-evolution is often analysed over very large time frames based on the evolutionary analysis across different species [188]. Nevertheless, genome-wide scans have identified several candidate loci that underlies local adaptations, which seems surprising given the short evolutionary time since the human divergence which is estimated have happened around 50,000 to 100,000 years ago when humans migrated out of Africa [188]. In light of this, it may make sense to analyse co-evolution within human populations based on the propositions that multiple genes within

a pathway or a functional sub-network may change in the same fitness direction at a same evolutionary rate to achieve a common phenotypic outcome [188]. In a study using data from the 1000 Genomes project [47] form East Asians, Europeans, and Africans populations, researchers found that genes having signals of recent positive selection are significantly closer to each other within protein-protein interaction (PPI) networks [188]. The approach was also able to identify known examples such as EGLN1 and EPAS1 (hypoxia-response pathway playing key roles in adaptation to high-altitude) as well as multiple genes in the NRG-ERBB4 (developmental) pathway [188]. This shows that sequences from shorter time spans can also be mined for co-evolution.

**MSA quality influences predictions** Since many co-evolutionary methods rely so heavily on multiple sequence alignments, it should not be surprising to know that the quality of the input alignment may affect the results. As one example, it is well known that structure-based alignment algorithms may be susceptible to shift error and other systematic errors, thus strong covariation signal can be caused by alignment errors leading to false positive predictions [70]. The phylogeny of the sequences also affects performance, since methods work better on large protein families having a wide but homogeneously distributed degree of sequence similarity ranging from distant to similar sequences [67]. In a recent study co-evolutionary methods applied to different alignments of the same protein family gave rise to distinct results, demonstrating that the measurement of co-evolution may greatly depend on the quality of the sequence alignment [70]. Even when alignments for the same protein family contained comparable numbers of sequences the number of estimated co-varying positions differed significantly. The authors of this analysis demonstrated that contact prediction can be improved by removing alignment errors due to several factors such as partial or otherwise erroneous sequences, the presence of paralogous sequences, and improper structure alignment.

**Co-Evolution and protein structure** Protein structure prediction from amino acid sequence is one of the ultimate goals in computational biology [30], despite significant efforts the general problem of *de novo* three-dimensional structure prediction has remained one of the most challenging problems in the field [156]. Unfortunately, *de-novo* protein structure prediction does not scale with longer proteins since the conformational space grows exponentially with the protein length. Inter-residue contact information can constrain the fold thus significantly reducing the search space. Since covariation patterns can complement experimental structural biology thus helping to elucidate functional interactions, information of co-evolutionary couplings between residues are often used to compute protein three-dimensional structures from amino acid sequences [156]. It has been observed that information about protein residue contacts, can be used to elucidate the fold of some proteins [119]. Researchers demonstrated that using co-evolutionary information from multiple sequence alignments greatly helps to deduce which amino acid pairs are close (or in contact) in the three-dimensional structure thus allowing the protein fold to be determined with a reasonable accuracy [156]. It is not surprising that the vast majority of methods for finding protein co-evolution are designed with the specific aim of generating results useful in the context of protein folding.

**Protein design** It has recently been proposed to use co-evolutionary theory in computational methods for protein design. Significant similarities were found between the amino acid covariation in natural protein sequences and sequences structures optimized by computational protein design methods [173]. Because evolutionary selective pressures on function and structure shaped the sequences to be close to optimal for their structures, natural protein sequences provide an excellent source for computational protein design methods. A study using computational protein design to quantify protein structure constraints from amino acid covariation for 40 diverse protein domains, shows that structural constraints imposed by covariation play a dominant role in protein architecture [173]. Thus, computational protein

design methods could make use of knowledge from natural co-evolution effects.

### 1.8.2 Global co-evolution models

Imagine a protein sequence of length  $L = a_1, a_2, \dots, a_n$ , amino acid  $a_i$  is coupled directly with  $a_j$ , and  $a_j$  to  $a_k$ , then  $a_i$  and  $a_k$  will show correlation despite not being directly coupled [243]. This is an important problem when inferring co-evolution as indirect coupling can make it difficult to recognize the directly co-evolving loci.

As opposed to models using the independence assumption, a ‘global’ model treats correlated pairs of residues as dependent on each other thereby minimizing effects of transitivity [156]. Since direct couplings are more reliable predictions of physical interactions, approaches that can distinguish direct from indirect couplings have been an intensive area of study [67]. Global approaches are designed to reach high scores only for amino acid pairs that are likely to be causative of the observed correlations [156]. In this section we introduce these methods.

**Glass spin systems** Global interaction models are well understood in statistical physics. A typical example are long-range order observed in spin systems, where the spins only have short-range direct interactions [20]. One of the first global models for co-evolution was proposed by Lapedes [138], who used a Monte Carlo algorithm to infer the simplest probabilistic distribution able to account for the whole network of covariations [67]. He presented a sequence-based probabilistic theory addressing co-operative effects in interacting positions in proteins assuming that a sequence of length  $L$  is a global state of an  $L$ -site spin system of twenty states (for twenty amino acids). Then he solved the global statistical formalism based on maximizing entropy under constraints which are known to lead to Boltzmann statistics [156]. Finally the conditional mutual information is calculated using this Boltzmann model which leads to the degree of covariation between residues at two positions factoring out contributions by interaction with the rest of the residues [156]. The amount sequence data is a limiting factor when performing inference of Boltzmann distribution parameters, thus it is

usually infeasible to use more than first order distributions [138]. Another limitation is the phylogenetic relatedness of these sequences, which is not addressed in this algorithm and has the potential to decrease accuracy [138].

**Direct coupling analysis** A similar approach called direct-coupling analysis (DCA) was also based on spin-glass physics [243]. In their implementation a generalized message-passing technique is used to massively parallelize the algorithm implementation. As in the work of Lapedes [138] an application of the maximum entropy principle yields the Boltzmann distribution which is used to estimate the second order interaction model. In principle higher correlations of three or more positions can be included, however dataset size (i.e. number of sequences in the MSA) does not allow for inference beyond two-residue model parameters. Determining model parameters, which is the most computationally expensive task is achieved by using a two-step procedure: i) given a candidate set of model parameters, single and two residue distributions are estimated; ii) the summation over all possible protein sequences would require  $O(|\Sigma_{AA}|^{N-2}N^2)$  steps (where  $\Sigma_{AA}$  is the amino acid alphabet and  $|\Sigma_{AA}|$  is the alphabet size), so an approximation is performed using MCMC sampling. This last step is the most expensive step and is expected to be very slow for 21-state variables. The message-passing approach implemented using an efficient heuristic, reduces the computational complexity to  $O(|\Sigma_{AA}|^2N^4)$ . Once all probability distributions are estimated, gradient descent is used to adjust the coupling strengths maximizing the joint probability of the data. Since the model is convex, it is guaranteed to converge to a single global maximum. Finally, a quantity called direct information (DI) measures the part of the mutual information of a position pair induced by the direct coupling (intuitively similar to mutual information in a two-variable model). Even after all optimizations and parallelizations, the method could not be applied to more than 60 positions in the protein alignment simultaneously. The authors apply the method to a dataset consisting of over 2,500 bacterial genes from a two-component signal transduction system,. Their global inference robustly

identified residue pairs proximal in space between sensor kinase (SK) and response regulator (RR) proteins as well as homo-interactions in RR proteins [243]. In their test dataset, the top 10 candidate interactions identified were shown to be true contacts, furthermore these predictions were then used to calculate an interacting protein complex quite accurately ( $3 \text{ \AA}$  RMSD) [243].

**Mean field approximation** DCA has been shown to yield a large number of correctly predicted contacts based on its ability to disentangle direct and indirect correlations; unfortunately the method is computationally expensive [243]. A method published by Morcos et al. [164] proposes a “mean field” approximation to DCA [243]. They first attempt to mitigate phylogenetic tree biases using a simple sampling correction based on re-weighting sequences with more than 80% similarity. In a nutshell, the approximation method also tries to disentangle direct and indirect couplings by inferring a global statistical and least-constrained model which, as discussed before, is achieved using a maximum-entropy principle leading to a Boltzmann distribution of couplings. The partition function ( $Z$ ) is then approximated by keeping only the linear order term in a Taylor series expansion, thus obtaining the mean-field equations. This approach is based on small-coupling expansion, thus a Taylor expansion around zero, a technique introduced in disordered Ising spin-glass models with binary variables. A well known result is that the first derivative of the Gibbs potential, the Legendre transform of the free energy  $F = -\ln(Z)$ , equals the average of the coupling term in the Hamiltonian. This simplifies this average calculation since the joint distribution of all variables becomes factorized over the single sites [164]. This mfDCA algorithm speeds up the original DCA implementation by  $10^3$  to  $10^4$  times [164], and can run on alignments up to 500 amino acids per row which is an order of magnitude larger the previous version of DCA based on message passing [164, 243].

**PSI-COV** Like other methods, PSI-COV [119] starts from a multiple sequence alignment. A covariance matrix is calculated by counting how often a given pair of amino acids occurs

in a particular pair of positions, summing over all sequences in the MSA. Since this matrix contains the raw data capturing all residue pair relationships, one can then compute a measure of causative correlations in the global statistical approaches by taking the inverse of the covariance matrix [119, 156]. Assuming that this covariance matrix can indeed be inverted, the inverse matrix relates to the degree of direct coupling, a well known fact in statistical theory under the assumption of continuous Gaussian multivariate distributions [156]. Elements significantly different from zero (off-diagonal) indicate pairs of sites which have strong direct coupling and are thus likely to be in direct physical contact [119]. Unfortunately, the empirical covariance matrices are actually almost always singular simply because it is unlikely that every amino acid is observed at every site. One of the most powerful techniques to overcome this problem is sparse inverse covariance estimation under Lasso constraints. The authors claim that the non-zero terms tend to accurately relate to correct correlations in the true inverse covariance matrix [119].

**Multidimensional mutual information** In a recent study a simple extension of mutual information was proposed by considering “additional information channels” corresponding to indirect amino acid dependencies [42]. This is achieved by defining the information  $I(X_1; X_3; X_2)$  representing an ‘interaction information’ for a channel with two inputs  $X_1$  and  $X_3$  and a single output  $X_2$ . The effect of the indirect input ( $X_3$ ) on the transmission between  $X_1$  and  $X_2$  can then be marginalized simply by summing mutual information for each possible value  $X_3$  weighted by the probability of occurrence [42]. Similarly a four variable model extension can be defined, in which case the marginalization would be done over two variables ( $X_3$  and  $X_4$ ). The authors test and compare their results using a set of 9 MSAs consisting of less than 400 sequences each, showing that their simple extension is comparable to other maximum entropy statistical models [42]. Even though the method is simple, the marginalization sums impose a heavy computational burden requiring long execution times and large memory footprints making the method impractical for sequences longer than 200

residues [42].

**Bayesian network model** Another attempt to disentangle direct from indirect statistical dependencies between residues assumes that the sequences in a MSA are drawn from unknown joint probability distribution [30]. The model considers pairwise conditional dependencies and factorizes the joint probability by a single other position which the residue depends on, using the conditional probabilities as nuisance parameters that are integrated out when calculating the likelihood of the alignment. Most notably, the model does not consider only the best way of choosing the dependent position, but rather sums over all possible ways in which dependencies could be chosen. This sum over all spanning trees is a generalization of Kirchhoff’s matrix-tree theorem and can be efficiently computed from the Laplacian of the dependency matrix.

### 1.8.3 Algorithm limitations

Mutual information was one of the first proposed methods used to detect covarying positions. As opposed to correlation-based methods, mutual information considers the distribution of each amino acid in the different sequences for a position quantifying whether presence of an amino acid one position can be used to predict presence of an amino acid in the other position. Mutual information does not take into account which amino acids are present, therefore different amino acids are treated just as symbols [67]. MI is an attractive and simple metric because it explicitly measures the dependence of one position on another, but it is limited by factors such as: i) positions with higher entropy (variability), tend to have higher MI than positions of lower entropy even though the latter are more constrained and would seem more likely to be co-evolving [73]; and ii) MI arises when alignments do not contain enough sequences to reduce the noise to signal ratio, it was shown that alignments should contain at least 125 sequences to significantly reduce this effect [157].

The influence of the background phylogenetic relationship between sequences in the MSA

confounds results and some efforts have tried to address this by removing certain problematic clades from the MSA. For instance, it has been shown that the effect may be limited to some degree by excluding highly similar sequences (from closely related species) from the alignment [249]. Continuous-time Markov process model for sequence coevolution can model this explicitly and some approaches have been implemented for small-scale studies of coevolution in small protein families, but computational limitations have hindered their usage in large-scale studies [67]. Other confounding effect is an uneven representation of protein sequence members (e.g. having several small subgroups and one large subgroup) which leads to statistical noise [156].

Since amino acids often contact more than one amino acids, transitive effects tend to form a network. Thus pairs of residues analysed using a simple statistical model (such as correlation or mutual information) may not necessarily be close in space or functionally constrained [156]. Algorithms to overcome this limitation exists, but they are based in global probabilistic models which require parameter estimation of complex distributions, such as the Boltzmann distribution, as well as marginalizing over all indirect variables. This makes global models computational prohibitively for all but very small datasets and impossible to apply to genome wide scale analysis.

Usually co-evolutionary methods are tested with high quality MSAs containing large number of sequences varying from  $5L$  to  $25L$  (where  $L$  is sequence length). Such large number of homogeneous sequences are rarely available and when they are, they usually correspond to well studied proteins and might already have a crystallized structure, thus analysis of amino acids in contact are not needed to infer the 3-D structure. Often, investigators study less well-characterized proteins having MSA of less than  $L$  sequences, and low alignment quality due to the presence of many gaps, in which case, existing methods are of limited value [42].

Finally it should be mentioned that results from different models usually do not agree, even for complex global models. In a recent study, a comparison of several methods shows that while all methods detected similar numbers of co-varying pairs , there is less than 65%

overlap between the top scoring prediction from methods based on different principles [42].

## 1.9 Thesis roadmap and Contributions

The original research presented in this thesis covers topics of computational and statistical methodologies related to the analysis of sequencing variants to unveil genetic links to complex disease. Broadly speaking, we address three types of problems: i) data processing of large datasets from high throughput biological experiments such as resequencing in the context of a GWAS (Chapter 2); ii) functional annotation of variants, i.e. calculating variant's impact at the molecular, cellular or even clinical level (Chapter 3); iii) identification of genetic risk factors for complex disease using models that combine population-level and evolutionary-level data to detect putative epistatic interactions (Chapter 4). When applicable, background material specific to each chapter is presented in a preface, together with an explanation of how that chapter ties in with the rest of the thesis.

This thesis comprises text and figures of articles that have either been published, submitted for publication, or ready to be submitted (waiting upon data embargo restrictions):

### Chapter 2

1. **P. Cingolani**, R. Sladek, and M. Blanchette. “BigDataScript: a scripting language for data pipelines.” *Bioinformatics* 31.1 (2015): 10-16.

For this paper, PC conceptualized the idea and performed the language design and implementation. RS & MB helped in designing robustness testing procedures. PC, RS & MB wrote the manuscript.

### Chapter 3

2. **P. Cingolani**, A. Platts, M. Coon, T. Nguyen, L. Wang, S.J. Land, X. Lu, D.M. Ruden, et al. “A program for annotating and predicting the effects of single

nucleotide polymorphisms, snpeff: Snps in the genome of drosophila melanogaster strain  $w^{1118}; iso - 2; iso - 3$ . Fly, 6(2), 2012.

For this paper, PC conceptualized the idea, implemented the program and performed testing. AP contributed several feature ideas, software testing and suggested improvements. XL, DR, SL, LW, TN, MC, LW performed mutagenesis and sequencing experiments. XL and DR performed the biological interpretation of the data. All authors contributed to the manuscript.

SnpEff's accompanying publication (SnpSift):

3. **P. Cingolani**, V. M. Patel, M. Coon, T. Nguyen, S. Land, D. M. Ruden, and X. Lu. “Using drosophila melanogaster as a model for genotoxic chemical mutational studies with a new program, snpsift”. Toxicogenomics in non-mammalian species, page 92, 2012.

We used SnpEff & SnpSift and developed a number of new functionalities in the context of two collaborative GWAS projects on type II diabetes:

4. M. McCarthy, T2D Genes Consortia. “Variation in protein-coding sequence and predisposition to type 2 diabetes”, Ready for submission.
5. A. Mahajan, X. Sim, H. Ng, A. Manning, M. Rivas, H. Heather, A. Locke, N. Grarup, H. K. Im, **P. Cingolani**, et al. “Identification and Functional Characterization of G6PC2 Coding Variants Influencing Glycemic Traits Define an Effector Transcript at the G6PC2-ABCB11 Locus.” PLoS genetics 11.1 (2015): e1004876-e1004876.

## Chapter 4

6. **P. Cingolani**, R. Sladek, and M. Blanchette. “A co-evolutionary approach for detecting epistatic interactions in genome-wide association studies”. Ready for submission (data embargo restrictions).

For this paper, PC designed the methodology under the supervision of MB and RS. PC implemented the algorithms. PC, RS & MB wrote the manuscript. This work uses data from the T2D consortia, thus it cannot be published until the main T2D paper is accepted for publication (according to T2D data embargo).

**Other contributions** During my thesis I have co-authored several other scientific articles (grouped by topic) published, submitted for publication, or ready to be submitted, not mentioned in this thesis:

## Epigenetics

7. **P. Cingolani**, X. Cao, R. Khetani, C.C. Chen, M. Coon, A. Bollig-Fischer, S. Land, Y. Huang, M. Hudson, M. Garfinkel, and others. “Intronic Non-CG DNA hydroxymethylation and alternative mRNA splicing in honey bees.” *BMC genomics* 14.1 (2013): 666.
8. M. Senut, A. Sen, **P. Cingolani**, A. Shaik, S. Land, Susan J and D. M. Ruden. “Lead exposure disrupts global DNA methylation in human embryonic stem cells and alters their neuronal differentiation.” *Toxicological Sciences* (2014).
9. D. M. Ruden, **P. Cingolani**, A. Sen, W. Qu, L. Wang, M. Senut, M. Garfinkel, V. Sollars, X. Lu, “Epigenetics as an answer to Darwin’s ‘special difficulty’ Part 2: Natural selection of metastable epialleles in honeybee castes”, *Frontiers in Genetics* (2015).
10. M. Senut, A. Sen, **P. Cingolani**, A. Shaik, S. Land, Susan J and D. M. Ruden. “Lead exposure induces changes in 5-hydroxymethylcytosine clusters in CpG islands in human embryonic stem cells and umbilical cord blood”, Submitted to ‘Epigenomics.
11. M. Senut, **P. Cingolani**, A. Sen, Arko, A. Kruger, A. Shaik, H. Hirsch, S. Suhr, D. Ruden. “Epigenetics of early-life lead exposure and effects on brain development.” *Epigenomics* 4.6 (2012): 665-674.

## GWAS & Disease

12. K. Oualkacha, Z. Dastani, R. Li, **P. Cingolani**, T. Spector, C. Hammond, J. Richards, A. Ciampi, C. Greenwood. “Adjusted sequence kernel association test for rare variants controlling for cryptic and family relatedness.” *Genetic epidemiology* 37.4 (2013): 366-376.
13. S. Bongfen, I. Rodrigue-Gervais, J. Berghout, S. Torre, **P. Cingolani**, S. Wiltshire, G. Leiva-Torres, L. Letourneau, R. Sladek, M. Blanchette, and others. “An N-ethyl-N-nitrosourea (ENU)-induced dominant negative mutation in the JAK3 kinase protects against cerebral malaria.” *PloS one* 7.2 (2012): e31012.
14. C. Meunier, L. Van Der Kraak, C. Turbide, N. Groulx, I. Labouba, Ingrid, **P. Cingolani**, M. Blanchette, G. Yeretssian, A. Mes-Masson, M. Saleh, and others. “Positional mapping and candidate gene analysis of the mouse Ccs3 locus that regulates differential susceptibility to carcinogen-induced colorectal cancer.” *PloS one* 8.3 (2013): e58733.
15. G. Caillard, G. Leiva-Torres, M. Leney-Greene, B. Charbonneau, A. Dumaine, N. Fodil-Cornu, M. Pyzik, **P. Cingolani**, J. Schwartzentruber, J. Dupaul-Chicoine, and others. “Genome-wide mouse mutagenesis reveals CD45-mediated T cell function as critical in protective immunity to HSV-1.” *PLoS pathogens* 9.9 (2013): e1003637.
16. M. Bouttier, D. Laperriere, M. Babak Memari, M. Verway, E. Mitchell, **P. Cingolani**, T. Wang, M. Behr, R. Sladek, M. Blanchette, S. Mader and J. White. “Genomics analysis reveals elevated LXR signaling reduces M. tuberculosis viability”, Submitted to *Journal of Clinical Investigation*.
17. M. Bouttier, D. Laperriere, M. Babak Memari, M. Verway, E. Mitchell, **P. Cingolani**, T. Wang, M. Behr, R. Sladek, M. Blanchette, S. Mader and J. White. “Genomic analysis of enhancers engaged in M. tuberculosis-infected macrophages

reveals that LXR signaling reduces mycobacterial burden”, Submitted to PLOS Pathogens.

### Fuzzy logic

18. **P. Cingolani** and Jesus Alcala-Fdez. “jFuzzyLogic: a robust and flexible Fuzzy-Logic inference system language implementation.” FUZZ-IEEE. 2012.
19. **P. Cingolani** and Jesus Alcala-Fdez. “jFuzzyLogic: a java library to design fuzzy logic controllers according to the standard for fuzzy control programming.” International Journal of Computational Intelligence Systems (2013), vol 6, pages 65-75.

# Chapter 2

## BigDataScript: A scripting language for data pipelines

### 2.1 Preface

The overall goal in this thesis is to find genetic loci related to complex disease. In order to have enough statistical power to find these risk loci, we need to sequence thousands of cases and controls (i.e. patients and healthy individuals). Obviously the first step is to find all these patients, obtain patients consent, take samples and keep track of clinically relevant variables (such as age, sex, BMI, and glycemic traits). Just by the sheer number of patients involved, its easy to see that the logistics are challenging, to say the least.

Once the sequencing of each patients DNA is performed, we need to process the raw sequencing information by performing what is known as “primary sequencing analysis”, which involves mapping reads to the reference genome, calling variants, as well as performing several types of quality controls. The term “primary analysis” makes it sound as if this step is simple, but it is not. Managing such volume of information is a huge task that requires large computational resources, and coordinating the process involved at every stage of the analysis is not trivial, even if the jobs are relatively easy to parallelize.

As an example of the complexity and data volumes involved in these analysis pipelines, mapping the raw reads to the reference genome (i.e. the first stage of the primary analysis) for our T2D sequencing data is estimated to take over 12,000 CPU hours, that is over 32 CPU/years, under the most optimistic assumptions. At this magnitude hardware and failures become a significant issue since the probability of one or more nodes malfunction while the data is being processed is quite high.

We designed and implemented a simple script-like programming language called BigDataScript (BDS), with a clean and minimalist syntax to develop and manage pipeline execution and provide robustness to various types of software and hardware failures as well as portability. This programming language specifically tailored for data processing pipelines, improves abstraction from hardware resources and assists with robustness. Hardware abstraction allows BDS pipelines to run without modification on a wide range of computer architectures, from a small laptop to multi-core servers, server farms, clusters, clouds or even whole datacenters. BDS achieves robustness by incorporating the concepts of absolute serialization and lazy processing, thus allowing pipelines to recover from errors. By abstracting pipeline concepts at programming language level, BDS simplifies implementation, execution and management of complex bioinformatics pipelines, resulting in reduced development and debugging cycles as well as cleaner code. BDS was used to create data analysis pipelines required for our research, including the ones described throughout this thesis, and is currently used by other research groups and sequencing facilities in both academic and private environments.

The rest of the chapter is published in: Cingolani, Pablo, Rob Sladek, and Mathieu Blanchette. “BigDataScript: a scripting language for data pipelines.” *Bioinformatics* 31.1 (2015): 10-16.

## 2.2 Introduction

Processing large amounts of data is becoming increasingly important and common in research environments as a consequence of technology improvements and reduced costs of high-throughput experiments. This is particularly the case for genomics research programs, where massive parallelization of microarray and sequencing-based assays can support complex genome-wide experiments involving tens or hundreds of thousands of patient samples [262]. With the democratization of high-throughput approaches and simplified access to processing resources (e.g. cloud computing), researchers must now routinely analyze large datasets. This paradigm shift with respect to the access and manipulation of information creates new challenges by requiring highly specialized skill, such as implementing data-processing pipelines, to be accessible to a much wider audience.

A data-processing pipeline, referred as “pipeline” for short, is a set of partially ordered computing tasks coordinated to process large amounts of data. Each of these tasks is designed to solve specific parts of a larger problem, and their coordinated outcomes are required to solve the problem as a whole. Many of the software tools used in pipelines that solve big data genomics problems are CPU, memory or I/O intensive and commonly run for several hours or even days. Creating and executing such pipelines require running and coordinating several of these tools to ensure proper data flow and error control from one analysis step to the next. For instance, a processing pipeline for a sequencing-based genome-wide association study may involve the following steps [12]: (i) mapping DNA sequence reads obtained from thousands of patients to a reference genome; (ii) identifying genetic changes present in each patient genome (known as “calling” variants); (iii) annotating these variants with respect to known gene transcripts or other genome landmarks; (iv) applying statistical analyses to identify genetic variants that are associated with differences in the patient phenotypes; and (v) quality control on each of the previous steps. Even though efficient tools exist to perform each of these steps, coordinating these processes in a scalable, robust and flexible pipeline is challenging because creating pipelines using general-purpose

computer languages (e.g. Java, Python or Shell scripting) involves handling many low-level process synchronization and scheduling details. As a result, process coordination usually depends on specific features of the underlying systems architecture, making pipelines difficult to migrate. For example, a processing pipeline designed for a “multi-core server” cannot directly be used on a cluster because running tasks on a cluster requires queuing them using cluster-specific commands (e.g. `qsub`). Therefore, if using such a language, programmers and researchers must spend significant efforts to deal with architecture-specific details that are not germane to the problem of interest, and pipelines have to be reprogrammed or adapted to run on other computer architectures. This is aggravated by the fact that the requirements change often and the software tools are constantly evolving.

In the context of bioinformatics, there are several frameworks to help implement data-processing pipelines; although a full comparison is beyond the scope of this article, we mention a few that relate to our work: (i) Snakemake (Koster and Rahmann, 2012) written as a Python domain-specific language (DSL), which has a strong influence from `make` command. Just as in `make`, the workflow is specified by rules, and dependencies are implied between one rules input files and another rules output files. (ii) Ruffus (Goodstadt, 2010), a Python library, uses a syntactic mechanism based on decorations. This approach tends to spread the pipeline structure throughout the code, making maintenance cumbersome [204]. (iii) Leaf [167], which is also written as a Python library, expresses pipelines as graphs drawn using ASCII characters. Although visually rich, the authors acknowledge that this representation is harder to maintain than the traditional code. (iv) Bpipe [204] is implemented as a DSL on top of Groovy, a Java Virtual Machine (JVM)-based language. Bpipe facilitates reordering, removing or adding pipeline stages, and thus, it is easy for running many variations of a pipeline. (v) NextFlow ([www.nextflow.io](http://www.nextflow.io)), another Groovy-based DSL, is based on data flow programming paradigm. This paradigm simplifies parallelism and lets the programmer focus on the coordination and synchronization of the processes by simply specifying their inputs and outputs.

Each of these systems creates either a framework or a DSL on a pre-existing general-purpose programming language. This has the obvious benefit of leveraging the languages power, expressiveness and speed, but it also means that the programmer may have to learn the new general-purpose programming language, which can be taxing and take time to master. Some of these pipeline tools use new syntactic structures or concepts (e.g. NextFlows data-flow programming model or Leafs pipeline drawings) that can be powerful, but require programming outside the traditional imperative model, and thus might create a steep learning curve.

In this article, we introduce a new pipeline programming language called BigDataScript (BDS), which is a scripting language designed for working with big data pipelines in system architectures of different sizes and capabilities. In contrast to existing frameworks, which extend general-purpose languages through libraries or DSLs, our approach helps to solve the typical challenges in pipeline programming by creating a simple yet powerful and flexible programming language. BDS tackles common problems in pipeline programming by transparently managing infrastructure and resources without requiring explicit code from the programmer, although allowing the programmer to remain in tight control of resources. It can be used to create robust pipelines by introducing mechanisms of lazy processing and absolute serialization, a concept similar to continuations (Reynolds, 1993) that helps to recover from several types of failures, thus improving robustness. BDS runs on any Unix-like environment (we currently provide Linux and OS.X pre-compiled binaries) and can be ported to other operating systems where a Java runtime and a GO compiler are available.

Unlike other efforts, BDS consists of a dedicated grammar with its own parser and interpreter, rather than being implemented on top of an existing language. Our language is similar to commonly used syntax and avoids inventing new syntactic structures or concepts. This results in a quick-to-learn, clean and minimalistic language. Furthermore, creating our own interpreter gives better control of pipeline execution and allows us to create features unavailable in general-purpose language (most notably, absolute serialization). This comes

at the expense of expressiveness and speed. BDS is not as powerful as Java or Python, and our simple interpreter cannot be compared with sophisticated just-in-time execution or JVM-optimized byte-code execution provided by other languages. Nonetheless, in our experience, most bioinformatics pipelines rely on simple programmatic constructs. Furthermore, in typical pipelines, the vast majority of the running time is spent executing external programs, making the executing time of the pipeline code itself a negligible factor. For these reasons, we argue that BDS offers a good trade-off between simplicity and expressiveness or speed.

## 2.3 Methods

In our experience, using general-purpose programming languages to develop pipelines is notably slow owing to many architecture-specific details the programmer has to deal with. Using an architecture agnostic language means that the pipeline can be developed and debugged on a regular desktop or laptop using a small sample dataset and deployed to a cluster to process large datasets without any code changes. This significantly reduces the time and effort required for development cycles. As BDS is intended to solve or simplify the main challenges in implementing, testing and programming data processing pipelines without introducing a steep learning curve, our main design goals are (i) simple programming language; (ii) abstraction from systems architecture; and (iii) robustness to hardware and software failure during computationally intensive data analysis tasks. In the next sections, we explore how these concepts are implemented in BDS.

### 2.3.1 Language overview

BDS is a scripting language whose syntax is similar to well-known imperative languages. BDS supports basic programming constructs (`if/ else`, `for`, `while`, etc.) and modularity constructs such as `functions` and `include` statements, which are complemented with architecture-

independent mechanisms for basic pipeline runtime control (such as task, sys, wait and checkpoint). At runtime, the BDS backend engine translates these high-level commands into the appropriate architecture-dependent instructions. At the moment, BDS does not support object-oriented programming, which is indeed supported by other pipeline tools based on libraries/DSL extending general-purpose programming languages. The complete language specification and documentation is available online at <http://pcingola.github.io/BigDataScript>.

Unlike most scripting languages, BDS is strongly typed, allowing detection of common type conversion errors at the initial parsing stage (pseudo-compilation) rather than at runtime (which can happen after several hours of execution). As the syntax of strict typing languages tends to be more verbose owing to longer variable declaration statements, we provide a type inference mechanism (operator `:=`) that improves code readability. For example (Listing 1), the variables `in` and `out` are automatically assigned the types the first time they are used (in this case, the type is assigned to be string).

### 2.3.2 Abstraction from resources

One of the key features of BDS is that it provides abstraction from most architecture-specific details. In the same way that high-level programming languages such as C or Java allow abstraction of the CPU type and other hardware features, BDS supports system-level abstraction, including the number and the type of computing-nodes or CPU-cores that are available to the pipeline and its component tasks, whether firing another process may saturate the servers memory or whether a process is executed immediately or queued.

Pipeline programming requires effective task management, particularly the ability to launch processes and wait for processes to finish execution before starting others. Task management can be performed using a single BDS statement, independently of whether this is running on a local computer or a cluster. Processes are executed using the `task` statement, which accepts an optional list of resources required by the task (for example, see Listing 1). The task consists of running a fictitious system command `myProcess` and diverting the

output to `output.file`. BDS currently supports the following architectures: (i) local, single or multi-core computer; (ii) cluster, using GridEngine, Torque and Moab; (iii) server farm, using ssh access; and (iv) cloud, using EC2 and StarCluster. Depending on the type of architecture on which the script is run, the task will be executed by calling the appropriate queuing command (for a cluster) or by launching it directly (for a multi-core server).

**Listing 2.1:** `pipeline.bds` program. A simple pipeline example featuring and a maximum of 6 h of execution time (Line 5).

---

```

1 ,  

2 #!/usr/bin/env bds  

3 in := “input.file”  

4 out := “output.file”  

5 task ( out <- in, cpus=2, timeout=6*HOUR ) {  

6   sys myProcess $in > $out # Invoke command  

}

```

---

BDS performs process monitoring or cluster queue monitoring to make sure all tasks end with a successful exit status and within required time limits. This is implemented using the `wait` command, which acts as a barrier to ensure that no statement is executed until all tasks finished successfully. Listing 2 shows a two-step pipeline with task dependencies using a `wait` statement (Line 13). If one or more of the `task` executions fail, BDS will wait until all remaining tasks finish and stop script execution at the `wait` statement. An implicit `wait` statement is added at the end of the main execution thread, which means that a BDS script does not finish execution until all tasks have finished running. It is common for pipelines to need multiple levels of parallel execution; this can be achieved using the `parallel` statement (or `par` for short). Wait statements accept a list of task IDs/parallel IDs in the current execution thread.

In addition to supporting explicitly defined task dependencies, BDS also automatically models implicit dependencies using a directed acyclic graph (DAG) that is inferred from information provided in the dependency operators (`<`) contained in `task` statements (see Listing 2, line 8). Finally, the `dep` expression defines a task whose conditions are not evalu-

ated immediately (as it happens in `task` expressions) but only executed if required to satisfy a `goal`. Using `dep` and `goal` makes it easier to define pipelines in a `declarative` manner that is similar to other pipeline tools, as tasks are executed only if the output needs to be updated with respect to the inputs, independent of the intermediate results file, which might have been deleted.

### 2.3.3 Robustness

BDS provides two different mechanisms that help create robust pipelines: lazy processing and absolute serialization. When a processing pipeline fails, BDS automatically cleans up all stale output files to ensure that rerunning the pipeline will produce a correct output. If a BDS program is interrupted, typically by pressing Ctrl-C on the console, all scheduled tasks and running jobs are terminated or deallocated from the cluster. In addition to immediately releasing computing resources, a clean stop means that users do not have to manually dequeue tasks, which allows them to focus on the problem at hand without having to worry about restoring a clean state.

**Lazy processing** Complex processing pipelines are bound to fail owing to unexpected reasons that range from data format problems to hardware failures. Rerunning a pipeline from scratch means wasting days on recalculating results that have already been processed. One common approach, when using general-purpose scripting languages, is to edit the script and comment out some steps to save processing time, which is inelegant and error prone. A better approach is to develop pipelines that incorporate the concept of lazy processing [167], a concept popularized by the `make` command (Feldman, 1979) used to compile programs, and which simply means the work is not done a task invoking a fictitious command `myProcess` defined to require 2 CPUs twice. This concept is at the core of many of the pipeline programming tools, such as SnakeMake, Ruffus, Leaf and Bpipe. By design, when lazy processing pipelines are rerun using the same dataset, they avoid unnecessary work. In

the extreme case, if a lazy processing pipeline is run on an already successfully processed dataset, it should not perform any processing at all.

BDS facilitates the creation of lazy processing pipelines by means of the dependency operator (`<-`) and conditional task execution (see Listing 1, line 5 for an example). The task is defined as `task (out < in)`, meaning that it is executed only if `out` file needs to be updated with respect to `in` file: for example, if `output.file` file does not exist, has zero length, is an empty directory or has been modified before `input.file`.

**Absolute serialization** This refers to the ability to save and recover a snapshot of the current execution state, compiled program, variables, scopes and program counter, a concept similar to continuations (Reynolds, 1993). BDS can perform an absolute serialization of the current running state and environment, producing checkpoint files from which the program can be re-executed, either on the same computer or on any other computer, exactly from the point where execution terminated. Checkpoint files (or `checkpoints` for short) also allow all variables and the execution stack to be inspected for debugging purposes (`bds -i checkpoint.chp`). The most common use of checkpoints is when a task execution fails. On reaching a `wait` statement, if one or more tasks have failed, BDS creates a checkpoint, reports the reasons for task execution failure and terminates. Using the checkpoint, pipeline execution can be resumed from the point where it terminated (in this case, at the most recently executed `wait` statement) and can properly re-execute pending tasks (i.e. the tasks that previously failed execution).

**Limitations** BDS is designed to afford robustness to the most common types of pipeline execution failures. However, events such as full cluster failures, emergency shutdowns, head node hardware failures or network problems isolating a subset of nodes may result in BDS being unable to exit cleanly, leading to an inconsistent pipeline state. These problems can be mitigated by a special purpose `checkpoint` statement that, as the name suggests, allows the programmer to explicitly create checkpoints. Given that the overhead of creating

checkpoints is minimal (a few milliseconds compared with hours of processing time for a typical pipeline), carefully crafted checkpoint statements within the pipeline code can be useful to prevent losing processed data, mitigate damage and minimize the overhead when rerun, which can be critical for long running pipelines.

### 2.3.4 Other features

Here we mention some selected features that are useful in pipeline programming. Extensive documentation is available at <http://pcingola.github.io/BigDataScript>.

**Automatic logging** Logging all actions performed in pipelines is important for three reasons: (i) it helps debugging; (ii) it improves repeatability; and (iii) it performs audits in cases where detailed documentation and logging are required by regulatory authorities (such as clinical trials).

Creating log files is simple, but it adds boilerplate code and increases the complexity of the pipeline. BDS performs automatic logging in three different ways. First, it directs all process StdOut/StdErr output to the console. Second, as having a single output can be confusing when dealing with thousands of processes running in parallel, BDS automatically logs each processs outputs (StdOut and StdErr) and exit codes in separate clearly identified files. Third, BDS creates a report showing both an overview and details of pipeline execution (Fig. 2.3).

**Automatic command line parsing** Programming flexible data pipelines often involves parsing command-line inputsa relatively simple but tedious task. BDS simplifies this task by automatically assigning values to variables specified through the command line. As an example, if the program in Listing 1 is called `pipeline.bds`, then invoking the program as `pipeline.bds -in another.file` will automatically replace the value of variable `in` with `another.file`.

Listing 2.2: `pipeline_2.bds` program. A two-step pipeline with task dependencies. The first step (line 9) requires to run `myProcess` command on a hundred input files, which can be executed in parallel. The second step (line 19) processes the output of those hundred files and creates a single output file (using fictitious `myProcessAll` command). It should be noted that we never explicitly state which hardware we are using: (i) if the pipeline is run on a dual-core computer, as each process requires 2 CPUs, one `myProcess` instance will be executed at the time until the 100 tasks are completed; (ii) if it is run on a 64-core server, then 32 `myProcess` instances will be executed in parallel; (iii) if it is run on a cluster, then 100 `myProcess` instances will be scheduled and the cluster resource management system will decide how to execute them; and (iv) if it is run on a single-core computer, execution will fail owing to lack of resources. Thus, the pipeline runs independent of the underlying architecture. The task defined in line 18 depends on all the outputs from tasks in line 8 (`mainOut <- outs`).

---

```

1  #!/usr/bin/env bds
2  // Step 1: Parallel processing of input files
3  string[] outs // Define a list of strings
4  for( int i=0 ; i < 100 ; i++ ) {
5      in := "input_$i.file"
6      out := "output_$i.file"
7      task ( out <- in, cpus=2, timeout=6*HOUR ) {
8          sys myProcess $in > $out
9      }
10     outs.add( out )           // Add all output files here
11 }
12 wait // Optional: Wait for all tasks to finish
13
14 // Step 2: Process all outputs from previous step
15 mainOut := "main.txt"
16 mainIn := outs.join(    ) // Create a string with all names (space-separated)
17 task ( mainOut <- outs, mem=10*G ) {
18     sys myProcessAll $mainIn > $mainOut
19 }
```

---

**Task re-execution** Tasks can be re-executed automatically on failure. The number of retries can be configured globally (as a command-line argument) or by a task (using the `retry` variable). Only after failing `retry+1` times will a task will be considered to have failed.

### 2.3.5 BDS implementation

BDS is programmed using Java and GO programming languages. Java is used for high-level actions, such as performing lexical analysis, parsing, creating abstract syntax trees (AST), controlling AST execution, serializing processes, queuing tasks, etc. Low-level details, such as process execution control, are programmed in GO. As BDS is intended to be used by programmers, it does not rely on graphical interfaces and does not require installation of complex dependencies or Web servers.

Figure 2.2 shows the cascade of events triggered when a BDS program is invoked. First the script `pipeline.bds` (Fig. 2.2A) is compiled to an AST structure (Fig. 2.2B) using ANTLR (Parr, 2007). After creating the AST, a runnable-AST (RAST) is created. RAST nodes are objects representing statements, expressions and blocks from our BDS implementation. These nodes can execute BDS code, serializing their state, and recover from a serialized file, thus achieving absolute serialization. The script is run by first creating a scope and then properly traversing the RAST (Fig. 2.2C). We note that if needed, this approach could be tuned to perform efficiently, as demonstrated by modern languages, such as Dart.

When recovering from a checkpoint, the scopes and RAST are deserialized (i.e. reconstructed from the file) and then traversed in recovery mode, meaning that the nodes do not execute BDS code. When the node that was executed at the time of serialization event is reached, BDS switches to run mode and the execution continues. This achieves execution recovery from the exact state at serialization time. Checkpoints are the full state of a programs instance and are intended as a recovery mechanism from a failed execution. This includes failures owing to corrupted or missing files, as BDS will re-execute all failed tasks

when recovering, thus correcting outputs from those tasks. However, checkpoints are not intended to recover from programming errors, where the user modifies the program to fix a bug, as a previously generated checkpoint is no longer valid respect to the new source code.

When a task statement is invoked, process requirements, such as memory, CPUs and timeouts, can optionally be specified. Depending on the architecture, BDS either checks that the underlying system has appropriate resources (CPUs and memory) to run the process (e.g. local computer or ssh-farm) or relies on the cluster management system to appropriately allocate the task. If all task requirements are met, a script file is created (Fig. 2.2D), and the task is executed by running an instance of bds-exec, a program that controls execution (Fig. 2.2E). This indirection is necessary for five reasons, which are described in detail below: (i) process identification, (ii) timeout enforcement, (iii) logging, (iv) exit status report and (v) signal handling.

**Process identification** means that bds-exec reports its process ID (PID), so that BDS can kill all child processes if the BDS script execution is terminated for some reason (e.g. the Ctrl-C key is pressed at the console).

**Timeout enforcement** has to be performed by bds-exec as many underlying systems do not have this capability (e.g. a process running on a server). When a timeout occurs, bds-exec sends a kill signal to all child processes and reports a timeout error exit status that propagates to the user terminal and log files.

**Logging** a process means that bds-exec redirects stdout and stderr to separate log files. These files are also monitored by the main BDS process, which shows the output on the console. As there might be thousands of processes running at the same time and operating systems have hard limits on the number of simultaneous file descriptors available for each user, opening all log files is not an option. To overcome this limit, BDS polls log file sizes, only opening and reading the ones that change.

**Exit status** has to be collected to make sure a process finished successfully. Unfortunately, there is no unified way to do this, and some cluster systems do not provide this

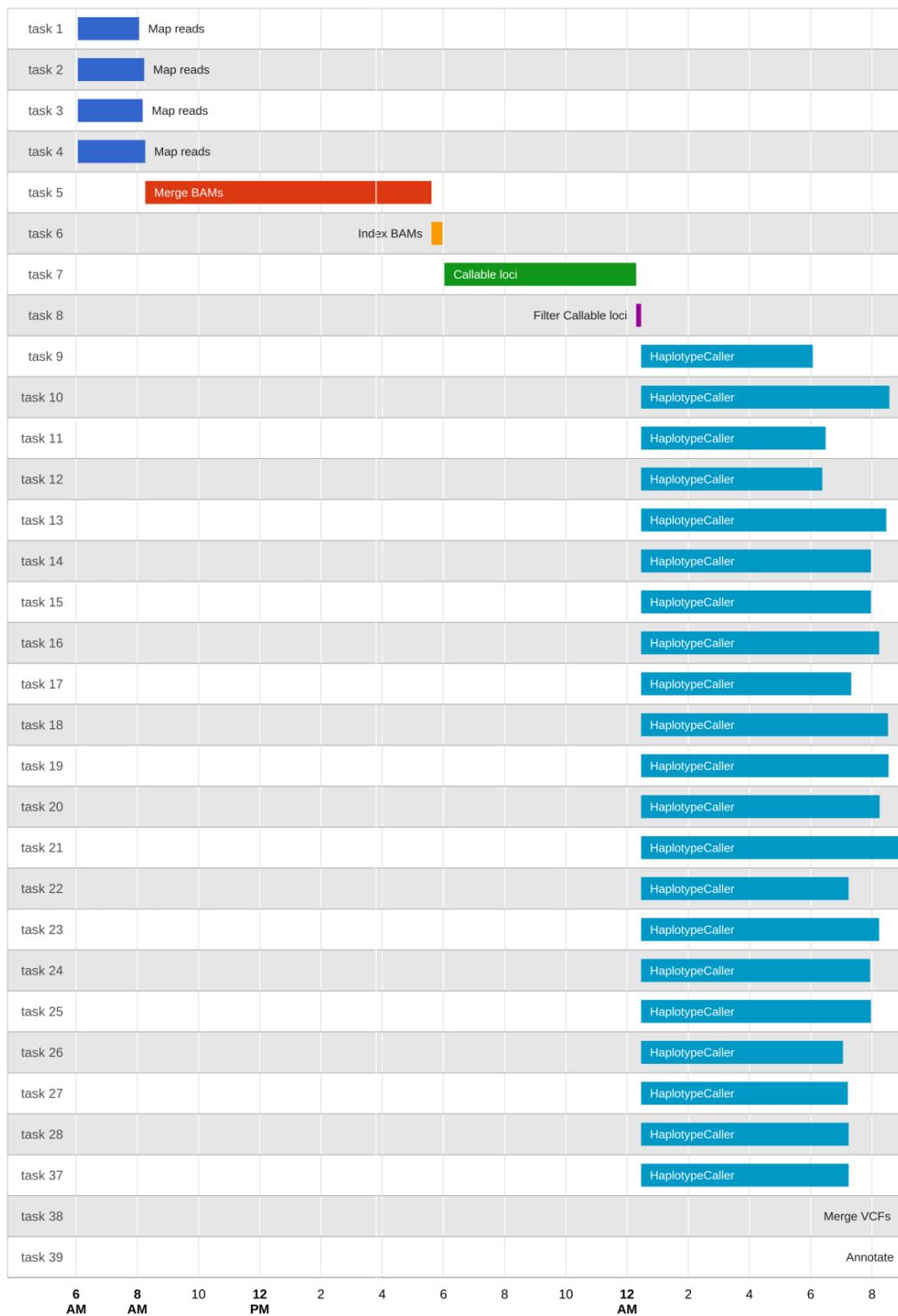


Figure 2.1: BDS report showing pipelines task execution timeline

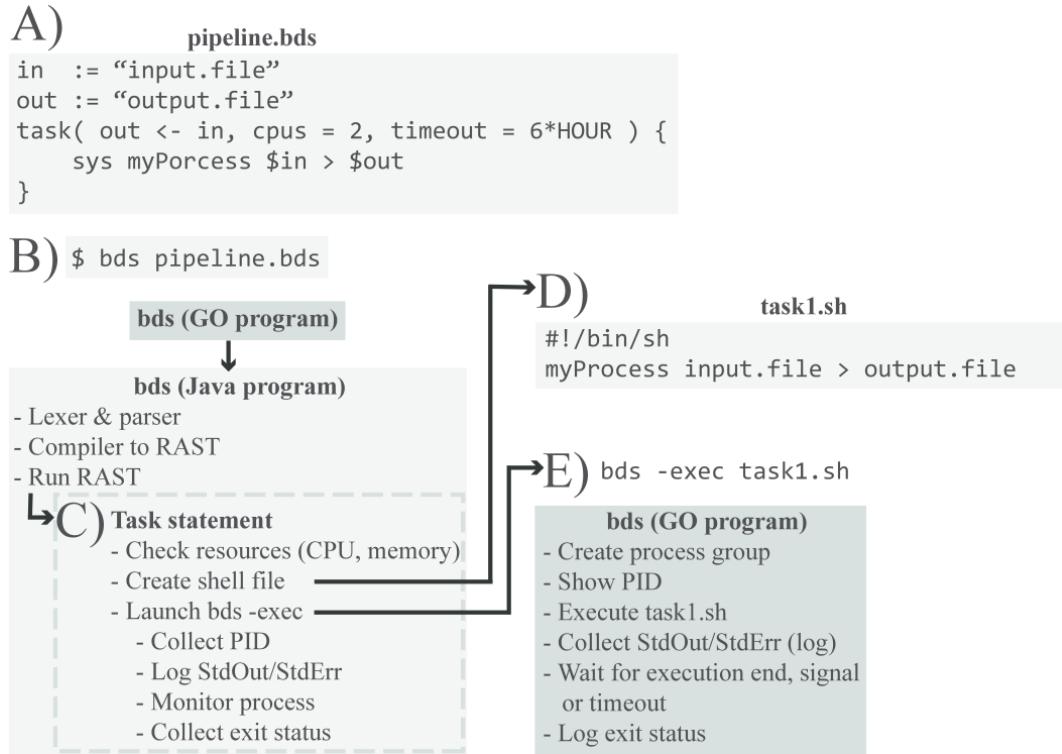


Figure 2.2: Execution example. (A) Script `pipeline.bds`. (B) The script is executed from a terminal. The GO executable invokes main BDS, written in JAVA, performs lexing, parsing, compilation to AST and runs AST. (C) When the task statement is run, appropriate checks are performed. (D) A shell script `task1.sh` is created, and a `bds-exec` process is fired. (E) `bds-exec` reports PID, executed the script `task1.sh` while capturing stdout and stderr as well as monitoring timeouts and OS signals. When a process finishes execution, the exit status is logged

information directly. By saving the exit status to a file, `bds-exec` achieves two goals: (i) unified exit status collection and (ii) exit status logging.

**Signal handling** is also enforced by `bds-exec` making sure that a kill signal correctly propagates to all subprocesses, but not to parent processes. This is necessary because there is no limit on the number of indirect processes that a task can run, and Unix/Posix systems do not provide a unified way to obtain all nested child processes. To be able to keep track of all subprocesses, `bds-exec` creates a process group and spawns the subprocess in it. When receiving a signal from the operating system, `bds-exec` traps the signal and propagates a kill signal to the process group.

## 2.4 Results

To illustrate the use of BDS in a real-life scenario, we present an implementation of a sequencing data analysis pipeline. This example illustrates three key BDS properties: architecture independence, robustness and scalability. The data we analyzed in this example consist of high-quality short-read sequences (200 coverage) of a human genome corresponding to a person of European ancestry from Utah (NA12877), downloaded from Illumina platinum genomes (<http://www.illumina.com/platinumgenomes>).

The example pipeline we created follows current best practices in sequencing data analysis [160], which involves the following steps: (i) map reads to a reference genome using BWA (Li and Durbin, 2009), (ii) call variants using GATKs HaplotypeCaller and (iii) annotate variants using SnpEff [39] and SnpSift [40]. The pipeline makes efficient use of computational resources by making sure tasks are parallelized whenever possible. Figure 2.3 shows a flowchart of our implementation, while the pipelines source code is available at `include/bio/seq` directory of our projects source code (<https://github.com/pcingola/BigDataScript>).

**Architecture independence** We ran the exact same BDS pipeline on (i) a laptop computer; (ii) a multi-core server (24 cores, 256 GB shared RAM); (iii) a server farm (5 servers, 2 cores each); (iv) a 1200-core cluster; and (v) the Amazon AWS Cloud computing infrastructure (Table 2.4). For the purpose of this example and to accommodate the fact that running the pipeline on a laptop using the entire dataset would be prohibitive, we limited our experiment to reads that map to chromosome 20. The architectures involved were based on different operating systems and spanned about three orders of magnitude in terms of the number of CPUs (from 4 to 1200) and RAM (from 8GB to 12TB). BDS can also create a cluster from a server farm by coordinating raw SSH connections to a set of computers. This minimalistic setup only requires that the computers have access to a shared disk, typically using NFS, which is a common practice in companies and university networks.

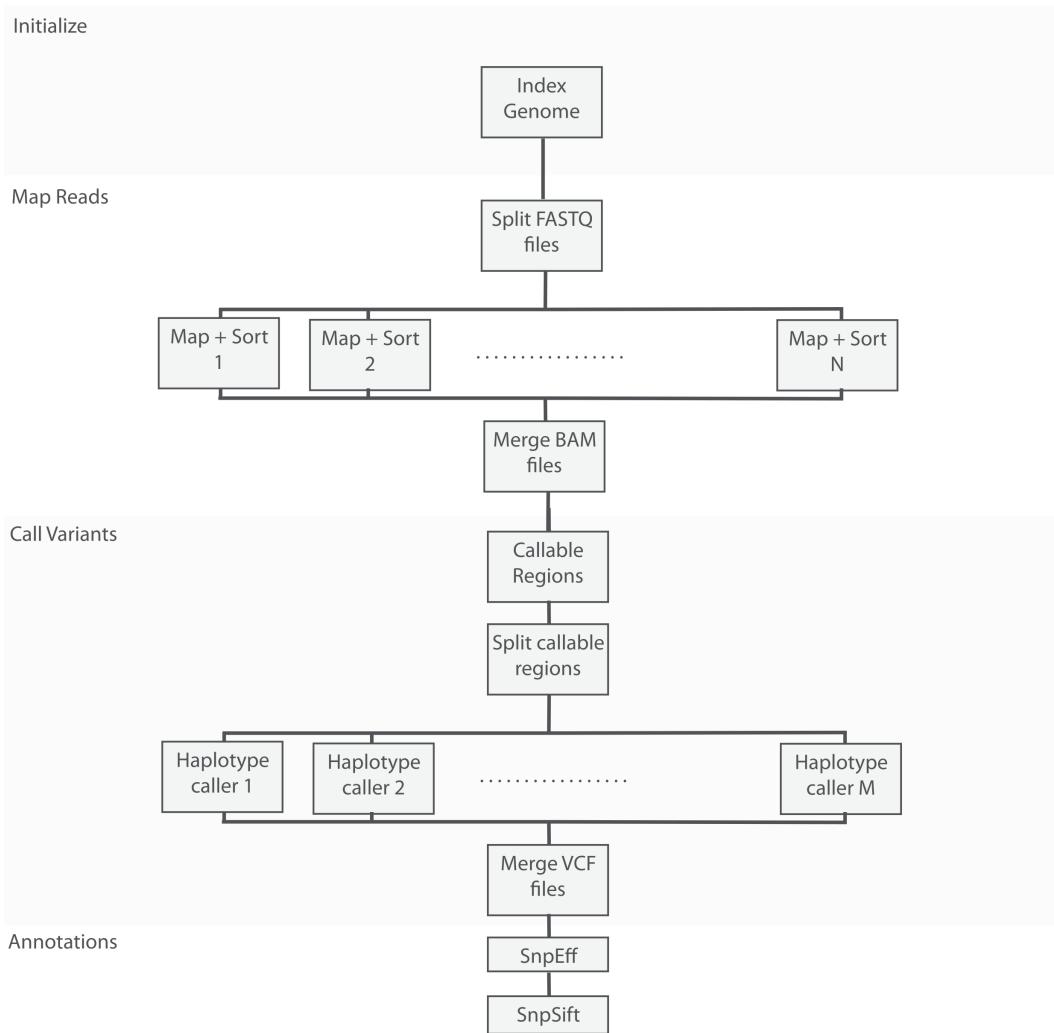


Figure 2.3: Whole-genome sequencing analysis pipeline's flow chart, showing how computations are split across many nodes

System	CPUs	RAM	Notes
Laptop (OS.X)	4	8 GB	
Server (Linux)	24	256 GB	
Server farm (ssh)	16	8 Gb	Server farm using 8 nodes, 2 cores each.
Cluster (PBS Torque)	1200	12 TB	High load cluster (over 95%).
Cluster (MOAB) (Random failures)	1200	12 TB	High load cluster (over 95%). Hardware induced failures.
Cloud (AWS + SGE)	Inf.	Inf.	StarCluster, 8 m1.large instances.

Table 2.4: Architecture independence example. Notes: Running the same BDS-based pipeline, a sequence variant calling and analysis pipeline, on the same dataset (chr20) but different architectures, operating systems and cluster management systems.

In all cases, the overhead required to run the BDS script itself accounted for 52 ms per task, which is negligible compared with typical pipeline runtimes of several hours.

**Robustness** To assess BDSs robustness, we ran the pipeline on a cluster where 10% of the nodes have induced hardware failures. As opposed to software failures, which are usually detected by cluster management systems, hardware node failures are typically more difficult to detect and recover from. In addition, we elevated the cluster load to 495% to make sure the pipeline was running on less than ideal conditions. As shown in Table 2.4, the pipeline finished successfully without any human intervention and required only 30% more time than in the ideal case scenario because BDS had to rerun several failed tasks. This shows how BDS pipelines can be robust and recover from multiple failures by using lazy processing and absolute serialization mechanisms.

**Scalability** To assess BDSs scalability, we ran exactly the same pipeline on two datasets that vary in size by several orders of magnitude (Table 2.5): (i) a relatively small dataset (chromosome 20 subset, 2GB) that would typically be used for development, testing and debugging and (ii) a high-depth whole-genome sequencing dataset (over 200 coverage, roughly 1.5 TB).

Dataset	Dataset size	System	CPUs	RAM
chr20	2 GB	Laptop (OS.X)	4	8 GB
Whole genome	1.5 TB	Cluster (MOAB)	22 000	80 TB

Table 2.5: Scaling dataset sized by a factor of 1000. Notes: The same sample pipeline run on dataset of 2 GB (reads mapping to human chromosome 20) and 1.5 TB (whole-genome data set). Computational times vary according to systems resources, utilization factor and induced hardware failures.

## 2.5 Discussion

We introduced BDS, a programming language that simplifies implementing, testing and debugging complex data analysis pipelines. BDS is intended to be used by programmers in a similar way to shell scripts, by providing glue for several tools to ensure that they execute in a coordinated way. Shell scripting was popularized when most personal computers had a single CPU and clusters or clouds did not exist. One can thus see BDS as extending the hardware abstraction concept to data-center level while retaining the simplicity of shell scripting.

BDS tackles common problems in pipeline programming by abstracting task management details at the programming language level. Task management is handled by two statements (`task` and `wait`) that hide system architecture details, leading to cleaner and more compact code than general-purpose languages. BDS also provides two complementary robustness mechanisms: lazy processing and absolute serialization.

A key feature is that being architecture agnostic, BDS allows users to code, test and debug big data analysis pipelines on different systems than the ones intended for full-scale data processing. One can thus develop a pipeline on a laptop and then run exactly the same code on a large cluster. BDS also provides mechanisms that eliminate many boilerplate programming tasks, which in our experience significantly reduce pipeline development times. BDS can also reduce CPU usage, by allowing the generation of code with fewer errors and

by allowing more efficient recovery from both software and hardware failures. These benefits generally far outweigh the minimal overhead incurred in typical pipelines.

# Chapter 3

A program for annotating and  
predicting the effects of single  
nucleotide polymorphisms, SnpEff:  
SNPs in the genome of Drosophila  
melanogaster strain  $w^{1118}; iso - 2; iso - 3$

## 3.1 Preface

As this thesis is focused on extracting biological insight from sequencing data, in this chapter we examine algorithms we created for calculating “functional annotations” of genomic variants. In essence, functional variant annotations are bits of biological knowledge that allow us to prioritize variants assumed to be relevant to the phenotypic trait under study and to filter out variants assumed irrelevant. The spectrum of functional annotations for a genomic variant is wide and may involve information on which genes are affected by the variant, how the protein product is affected, how conserved is the genomic region the variant lies onto,

and which clinically relevant information is associated with the loci; just to mention a few typical use cases.

When trying to find variants that affect risk of complex disease, statistical power is paramount. We need to be able to “separate wheat from chaff”. In our context this means two different but closely related tasks: i) performing functional annotations, and ii) using that information for prioritizing variants (and filtering out the ones we suspect are not related to the particular trait under study). Failing to efficiently filter out irrelevant variants would reduce our statistical power as more statistical tests are calculated, thus would decrease our chances of finding the associations we are looking for. In order to efficiently annotate and filter variants, we created two software packages called SnpEff and SnpSift that deal with the annotation and filtering aspects respectively.

The rest of the chapter is published in: **P. Cingolani**, A. Platts, M. Coon, T. Nguyen, L. Wang, S.J. Land, X. Lu, D.M. Ruden, et al. “A program for annotating and predicting the effects of single nucleotide polymorphisms, snpeff: Snps in the genome of drosophila melanogaster strain *w<sup>1118</sup>; iso – 2; iso – 3*”. Fly, 6(2), 2012.

## 3.2 Abstract

We describe a new computer program, SnpEff, for rapidly categorizing the effects of single nucleotide polymorphisms (SNPs) and other variants such as multiple nucleotide polymorphism (MNPs) and insertion-deletions (InDels), in whole genome sequences. Once a genome is sequenced, the SnpEff program can be used to annotate and classify genetic polymorphisms based on their effects on annotated genes, such as synonymous or non-synonymous SNPs, start codon gains or losses, stop codon gains or losses; or based on their genomic locations, such as intronic, 5' untranslated region (5' UTR), 3' UTR, upstream, downstream or intergenic regions. Here the use of SnpEff is illustrated by annotating  $\tilde{3}56,660$  candidate SNPs in  $\tilde{1}17$  Mb unique sequences, representing a substitution rate of  $\tilde{1}/305$  nucleotides, between the

*Drosophila melanogaster*  $w^{1118}; iso - 2; iso - 3$  strain and the reference  $y^1; cn^1bw^1sp^1$  strain [183]. We show that  $\tilde{1}5,842$  SNPs are synonymous and  $\tilde{4},467$  SNPs are non-synonymous ( $N/S \tilde{0.28}$ ) and the remainder are in other categories, such as stop codon gains (38 SNPs), stop codon losses (8 SNPs) and start codon gains (297 SNPs) in the 5' UTR. We found, as expected, that the SNP frequency is proportional to the recombination frequency (i.e., highest in the middle of chromosome arms). We also found that start-gained and stop-lost SNPs in *Drosophila melanogaster* often encode N-terminal and C-terminal amino acids that are conserved in other *Drosophila* species. This suggests that the 5' and 3' UTRs are reservoirs of cryptic genetic variation that can be used multiple times during the evolution of the *Drosophila* genus. At this time, SnpEff has been set up for annotating DNA polymorphisms of over 320 genome versions of multiple species including the human genome. It has already been used by over 50 institutions and universities in the bioinformatics community. Tools such as SnpEff are valuable because, as sequencing becomes cheaper and more available, whole genome sequencing is becoming more important in model organism genetics.

### 3.3 Introduction

When we re-sequenced the  $w^{1118}; iso - 2; iso - 3$  genome in 2009, [183] bioinformatics tools available then were unable to rapidly categorize the  $\tilde{3}56,660$  SNPs as comparing to the  $y^1; cn^1bw^1sp^1$  reference strain. At the time, other available tools such as ENSEMBLs variant web application (<http://ensembl.org>) could only analyze a few hundred to a few thousand SNPs per batch. Therefore, over the past couple of years, we have been developing a new program, SnpEff, which is able to analyze and annotate thousands of variants per second. In addition to SnpEff, other programs to annotate genomic variants are currently now available, such as Annotate Variation (ANNOVAR) [238] and Variant Annotation, Analysis and Search Tool (VAAST) [200]. However, SnpEff supports more genome versions, is open source for any user, supports variant call format (VCF) files and it is marginally faster (although the speeds

# SNP	Gene_name	Effect	Old_AA/new_AA	Old_codon/New_codon	Codon_Num (CDS)	CDS_size
chr2L:10006682_C/T	CG31755	UPSTREAM: 541 bases				
chr2L:10006758_G/A	CG31755	UPSTREAM: 465 bases				
chr2L:10007289_G/A	CG4747	SYNONYMOUS_CODING	L/L	TTG/TTA	489	1809
chr2L:10007319_G/C	CG4747	SYNONYMOUS_CODING	G/G	GGG/GGC	499	1809
chr2L:10007356_A/T	CG4747	INTRON				1809
chr2L:10007363_T/A	CG4747	INTRON				1809

Table 3.1: Output of SnpEff. # SNP, a description of the single nucleotide polymorphism (SNP) indicating chromosome arm (chr2L), coordinate in genome (10006682), and nucleotide change (e.g., C/T indicates that C is replaced by T in  $w^{1118}; iso - 2; iso - 3$  at this position). Gene\_name, official gene symbol of gene. Effect, description of SNP (e.g., upstream of transcription start site at position 541). Old\_AA/new\_AA, amino acid change, if any, in one letter code. Old\_codon/New\_codon, if a codon contains a SNP, the old (reference) and new ( $w^{1118}; iso - 2; iso - 3$ ) codons are indicated. Codon\_Num (CDS), the codon number of the coding sequence (CDS). CDS\_size, the size of the protein in amino acids.

of SnpEff, ANNOVAR and VAAST are comparable). Table S1 shows a feature comparison of some currently available software packages.

SnpEff, an abbreviation of “SNP effect,” is a multi-platform open source variant effect predictor program. SnpEff annotates variants and predicts the coding effects of genetic variations, such as SNPs, insertions and deletions (INDELs) and multiple nucleotide polymorphisms (MNPs) (<http://SnpEff.sourceforge.net/>). The main features of SnpEff include: (1) speedthe ability to make thousands of predictions per second; (2) flexibilitythe ability to add custom genomes and annotations; (3) the ability to integrate with Galaxy, an open access and web-based platform for computational bioinformatic research (<http://gmod.org/wiki/Galaxy>); (4) compatibility with multiple species and multiple codon usage tables (e.g., mitochondrial genomes); (5) integration with Genome Analysis Toolkit (GATK) [160]; and (6) ability to perform non-coding annotations. When SnpEff was integrated into the GATK, it replaced the ANNOVAR program for variant analyses.

A simple walk-through example on how to analyze sequencing data to calculate variants and their effects is shown in Listing SL1. This example is intended for illustration purposes only since many additional steps are routinely used in re-sequencing data analysis pipelines, but design of a fully featured pipeline is beyond the scope of this paper.

Here, we report the results of SnpEff (version 1.9.6) analyses of the ~356,660 candidate SNPs that we identified in  $w^{1118}; iso - 2; iso - 3$  with respect to the  $y^1; cn^1bw^1sp^1$  reference strain as reported in our previous paper.<sup>1</sup> This is of great interest to the Drosophila community because thousands of transposon insertion stocks [226] and hundreds of deficiency stocks [176],[176] were generated in the  $w^{1118}; iso - 2; iso - 3$  genetic background. The large number and potential severity of many SNPs in the two laboratory strains was a surprising finding, and the possible evolutionary implications of this finding are discussed.

## 3.4 Results

**Formats used in SnpEff.** To understand the potential effects of large numbers of SNPs in genome sequence comparisons, we developed an open-source tool, SNPEff, to classify SNPs based on gene annotations. Table 3.1 shows the beginning portion of the output generated by SnpEff when the SNPs in  $w^{1118}; iso - 2; iso - 3$  were compared with the reference genome,  $y^1; cn^1bw^1sp^1$  that is represented in Drosophila melanogaster release 5.3. A more complete SnpEff effect list is shown in Table 3.2. Before using SnpEff, an input file must be generated that lists all of the SNPs and INDELS in a genome. We published the input file for  $w^{1118}; iso - 2; iso - 3$  in our previous paper,<sup>1</sup> and it was derived by comparing hundreds of millions of short sequence reads (20-fold genome coverage) and identifying SNPs based on a Sequence Alignment/Map tools (SAMtools) quality score for each nucleotide in the genome [142].

Input formats supported by SnpEff are variant call format (VCF) [61], tab separated TXT format; and the SAMtools

Pileup format [142]. VCF was created by the 1,000 Genomes project and it is currently the de facto standard for variants in sequencing applications. The TXT and Pileup formats are currently deprecated and being phased out.

SnpEff also supports two output formats, TXT and VCF. The information provided

in both of them includes four main groups: (i) variant information (genomic position, the reference and variant sequences, change type, heterozygosity, quality and coverage); (ii) genetic information (gene Id, gene name, gene biotype, transcript ID, exon ID, exon rank); and (iii) effect information (effect type, amino acid changes, codon changes, codon number in CDS, codon degeneracy, etc.).

Whenever multiple transcripts for a gene exist, the effect and annotations on each transcript are reported, so one variant can have multiple output lines. Table 3.3 shows the information provided by each column in TXT format and Table 3.4 shows the information provided in VCF format. When using VCF format, the effect information is added to the information (INFO) fields using an effect (EFF) tag. As in the case of TXT output, if multiple alternative splicing products are annotated for a particular gene, SnpEff provides this information for each annotated version (see Sup. Data File 1 for the complete SnpEff output for  $w^{1118}; iso - 2; iso - 3$ ).

Predicted effects are with respect to protein coding genes. Variants affecting non-coding genes are annotated and the corresponding biotype is identified, whenever the information is available. A “biotype” is a group of organisms having the same specific genotype.

According to SnpEff (version 1.9.6), the largest number of SNPs in  $w^{1118}; iso - 2; iso - 3$  are in introns (130,126) followed by those in upstream (76,155), downstream (71,645) and intergenic (51,783) regions (Fig. 3.5). “Upstream” is defined as 5 kilobase (kb) upstream of the most distal transcription start site and “downstream” is defined as 5 kb downstream of the most distal polyA addition site, but these default variables can be easily adjusted. SnpEff also found thousands of SNPs within the exons. For example, there are 3,718 SNPs in the 3’ untranslated regions (3’ UTR) and 2,508 SNPs in the 5’ untranslated regions (5’ UTR). The SNPs in the upstream, downstream, 5’ and 3’ UTR regions might affect transcription or translation, but the actual effects have to be confirmed case-by-case. In the next few sections, we present examples of several types of SNPs that might affect the protein function.

Heterozygosity is not considered in the  $w^{1118}; iso - 2; iso - 3$  sequence because the stock

Effect	Note
INTERGENIC	The variant is in an intergenic region
UPSTREAM	Upstream of a gene (default length: 5K bases)
UTR_5_PRIME	Variant hits 5'UTR region
UTR_5_DELETED	The variant deletes and exon which is in the 5'UTR of the transcript
START_GAINED	A variant in 5'UTR region produces a three base sequence that can be a START codon
SPLICE_SITE_ACCEPTOR	The variant hits a splice acceptor site (defined as two bases before exon start, except for the first exon)
SPLICE_SITE_DONOR	The variant hits a Splice donor site (defined as two bases after coding exon end, except for the last exon)
START_LOST	Variant causes start codon to be mutated into a non-start codon
SYNONYMOUS_START	Variant causes start codon to be mutated into another start codon
CDS	The variant hits a CDS
GENE	The variant hits a gene
TRANSCRIPT	The variant hits a transcript
EXON	The variant hits an exon
EXON_DELETED	A deletion removes the whole exon
NON_SYNONYMOUS_CODING	Variant causes a codon that produces a different amino acid
SYNONYMOUS_CODING	Variant causes a codon that produces the same amino acid
FRAME_SHIFT	Insertion or deletion causes a frame shift
CODON_CHANGE	One or many codons are changed
CODON_INSERTION	One or many codons are inserted
CODON_CHANGE_PLUS_CODON_INSERTION	One codon is changed and one or many codons are inserted
CODON_DELETION	One or many codons are deleted
CODON_CHANGE_PLUS_CODON_DELETION	One codon is changed and one or more codons are deleted
STOP_GAINED	Variant causes a STOP codon
SYNONYMOUS_STOP	Variant causes stop codon to be mutated into another stop codon
STOP_LOST	Variant causes stop codon to be mutated into a non-stop codon
INTRON	Variant hits and intron. Technically, hits no exon in the transcript
UTR_3_PRIME	Variant hits 3'UTR region
UTR_3_DELETED	The variant deletes and exon which is in the 3'UTR of the transcript
DOWNSTREAM	Downstream of a gene (default length: 5K bases)
INTRON_CONSERVED	The variant is in a highly conserved intronic region
INTERGENIC_CONSERVED	The variant is in a highly conserved intergenic region

Table 3.2: Detailed effect list from SnpEff

was isogenized and only high quality (i.e., homozygous SNPs) were used for this analysis. 1

The SnpEff website (<http://snpeff.sourceforge.net/SnpSift.html>) has a frequently asked questions (FAQ) section that addresses most issues that a user might have in operating this program.

**SNPs that generate new start codons.** There are 297 SNPs that potentially generate a new translation initiation codon in the 5' UTR (start-gained SNPs). The most common translation initiation codon is AUG, which is coded by ATG in the genome. To be thorough, we also included CUG and UUG codons, which code for leucine, as these codons can also be used to initiate translation in rare genes in *Drosophila* and mammals [224],[115]. There are 60 genes with ATG start-gained SNPs (Table 3.6), 99 genes with CTG start-gained SNPs and 120 genes with TTG startgained SNPs in *w<sup>1118</sup>; iso-2; iso-3*, all by definition in 5' UTR regions, compared with the reference genome (the reading frame is indicated on the SnpEff table). Most of the ATG start-gained SNPs are within 1 kb of the annotated translation start (Table 3.6), but this probably reflects the fact that most 5' UTR sequences are less than 1 kb long. Less than expected by chance, only ~25% of the ATG start-gain SNPs are in the same reading frame as the annotated translation start point (Table 3.6). Since 33% of in frame ATG start-gained SNPs are expected by chance, this suggests that there might be weak selection against this class of SNPs. Of the 60 genes with ATG start-gained SNPs, five genes have two ATG start-gained SNPs and one gene has three startgained SNPs; the remaining 54 genes have a single start-gained SNP. Since SnpEff does not take into account the Kozak consensus sequence flanking the AUG site, 5'-ACC AUG G-3', that is generally required for efficient translation [130], and thus further assessment is required to determine whether a start-gained SNP is actually used.

Gene ontology (GO) pathway analysis of the genes affected by the 297 start-gain SNPs in *w<sup>1118</sup>; iso-2; iso-3* was done using DAVID (Database for Annotation, Visualization and Integrated Discovery) [68, 110]. We found that the GO categories “tissue morphogenesis,” “immunoglobulin like,” “developmental protein,” and “alternative splicing” are significantly

Column	Notes
Chromosome	Chromosome name (usually without any leading 'chr' string)
Position	One based position
Reference	Reference
Change	Sequence change
Change type	Type of change (SNP, MNP, INS, DEL)
Homozygous	Is this homozygous or heterozygous (Hom, Het)
Quality	Quality score (from input file)
Coverage	Coverage (from input file)
Warnings	Any warnings or errors.
Gene_ID	Gene ID (usually ENSEMBL)
Gene_name	Gene name
Bio_type	BioType, as reported by ENSEMBL
Trancript_ID	Transcript ID (usually ENSEMBL)
Exon_ID	Exon ID (usually ENSEMBL)
Exon_Rank	Exon number on a transcript
Effect	Effect of this variant. See details below
old_AA/new_AA	Amino acid change
old_codon/new_codon	Codon change
Codon_Num(CDS)	Codon number in CDS
Codon_degeneracy	Codon degeneracy
CDS_size	CDS size in bases
Custom_interval_ID	If any custom interval was used, add the IDs here (may be more than one)

Table 3.3: Information provided by SnpEff in tab separated output format (TXT)

enriched after multiplecomparisons correction by false-discovery rate (FDR  $\leq$  0.001; Table 3.7). These categories are interesting because they predominantly contain proteins that show a wide degree of intra- and interspecies variability. For example, the immunoglobulin loci, which are highly divergent among humans and in other vertebrates, are used for antigen recognition [140]. Also, developmental proteins and proteins involved in tissue morphogenesis often have both conserved domains, such as the Hox domain, and highly divergent domains that maintain morphological diversity within a species, such as the trans-activation domains [201, 99].

Our previous analyses suggest that most of the SNPs that we identified in *w<sup>1118</sup>;iso-2;iso-3* are probably genuine and can be validated by capillary sequencing. A common worry about nextgeneration sequencing data in general is that SNPs are vastly over estimated. One might think that if a large fraction of the identified SNPs had the predicted “effects”, the organism would not be viable. However, since short-read next-generation sequencing has a high error rate, such as the short-read sequences we obtained with the Illumina platform, further validation of specific SNPs is needed to be absolutely certain. Further validation of SNPs is best done with long-range DNA sequencing, such as with traditional capillary sequencing, or sequencing with the Roche [244], and many other DNA sequencing instruments that are now available [208] (see [183] for validation examples with capillary sequencing).

An example of a start-gained SNP is found in the 5' UTR of Ecdysone inducible protein 63E (Eip63E) gene, which is predicted to be a cyclin J dependent kinase required for oogenesis and embryonic development (Fig. 3.8) [149]. The potential start-gain SNP (A  $\rightarrow$  G) in Eip63E changes 5'-ATA-3' to 5'-ATG-3' in the same reading frame with no in-frame intervening stop codons (Fig. 3.8A). If translation occurs at the new start-gained SNP, it would produce a protein with 57 additional N-terminal amino acids compared with the reference gene (Fig. 3.8B). However, the three bases prior to the new 5'-ATG-3' sequence, 5'-AAT-3', is a poor match to the Kozak consensus sequence, 5'-ACC-3', discussed above in

reference 12. Therefore, it is unclear whether the startgain SNP in Eip63E is recognized by the ribosomal machinery.

It is interesting that a BLASTp search of the protein database reveals that the N-terminal 57 amino acids in Eip63E are 63% identical (36/57) to the 58 N-terminal amino acids of the orthologous gene in *Drosophila yakuba*, but not to any other *Drosophila* species. *D. yakuba* is very close to *D. melanogaster* in the phylogeny. This suggests that the 5' UTR of Eip63E might be a source for cryptic genetic variation encoding novel N-terminal protein sequences that potentially modulates protein function (see Discussion).

**SNPs that generate new stop codons.** Another surprise in our SnpEff analysis was the identification of 28 stop-gained SNPs and 5 stop-lost SNPs in *w<sup>1118</sup>; iso - 2; iso - 3* (Table 3.9). A stop-gained SNP, classically called a nonsense SNP, has a coding codon changed to a stop codon, UAA, UAG, UGA [26]. Three genes, *oc/ otd*, *LRP1* and *trol9*, have two stop-gained SNPs. Surprisingly at least 8 of the stop-gained SNPs are in genes that encode essential proteins, and these are *Dif*, *dp*, *ex*, *MESR4*, *mew*, *oc/ otd*, *tai* and *trol*. It is not known whether the other stop-gained SNPs also affect essential protein-coding genes because their functions have not yet been characterized (according to [www.flybase.org](http://www.flybase.org)). We note that what would be a stop-gained SNP in *w<sup>1118</sup>; iso - 2; iso - 3* would be a stop-lost SNP in the reference strain, and vice versa, because the sequence of the ancestral *Drosophila melanogaster* strain that gave rise to both of these strains is not known.

An important consideration with stop-gained and stop-lost SNPs is whether the C-terminal amino acids in the longest version of the protein that not present in the shortest version of the protein are conserved in other *Drosophila* species. If the additional C-terminal amino acids are not conserved, then these amino acids might not affect the essential function of the protein but they might exert modulatory effects. If the additional C-terminal amino acids are conserved in multiple *Drosophila* species, then their loss might adversely affect the function of the protein. Therefore, in Table 3.9, we further classify the stop-gained and stop-lost SNPs into four categories: Category 1, including 23 genes, with both the N-terminal

Sub-field	Notes
Effect	Effect of this variant. See details below
Codon_Change	Codon change: old_codon/new_codon
Amino_Acid_change	Amino acid change: old_AA/new_AA
Warnings	Any warnings or errors
Gene_name	Gene name
Gene_BioType	BioType, as reported by ENSEMBL
Coding	[CODING   NON_CODING]. If information reported by ENSEMBL (e.g., has 'protein_id' information in GTF file)
Trancipt	Transcript ID (usually ENSEMBL)
Exon	Exon ID (usually ENSEMBL)
Warnings	Any warnings or errors (not shown if empty)

Table 3.4: Information provided by SnpEff in variant call format (VCF). The information is added to the INFO fields using an tag 'EFF'. The format for each effect is “Effect (Effect\_Impact | Codon\_Change | Amino\_Acid\_change | Gene\_Name | Gene\_BioType | Coding | Transcript | Exon [ | ERRORS | WARNINGS ] )”.

and novel C-terminal regions conserved among Drosophila species and other organisms; Category 2, including only one gene, with the entire gene sequence not conserved even among other Drosophila species; Category 3, with two genes, with the novel C-termini not conserved among other Drosophila species. In this category, the N-termini are conserved among Drosophila species, but this conservation is not maintained beyond the Drosophila genus (this class is likely a novel gene that arose in the Drosophila genus); and Category 4, including seven genes, with the novel C-terminal regions conserved among other Drosophila species but not beyond the Drosophila genus. In this category, the N-terminus is conserved beyond the Drosophila genus (this class probably has a C-terminal domain with a modulatory role in the Drosophila genus but not beyond the genus).

An example of an essential protein-coding gene in Category 4, where the novel C-terminus is not conserved outside the Drosophila genus, is *oceliless* (*oc*), also known as *orthodenticle* (*otd*) (Fig. 3.10). The *oc/otd* gene has two in-frame stop-gained SNPs in *w<sup>1118</sup>;iso-2;iso-3*. The *oc/otd* gene is a Hox-family transcription factor required for photoreceptor development in the compound eye and the light-sensing ocellus, embryonic development and brain segmentation [1, 255]. The Hox domain is 60 amino acids, 59 of which are identical with the human Otd protein. The Hox domains, which arose before invertebrates and vertebrates split several hundred million years ago, are among the most conserved protein domains in

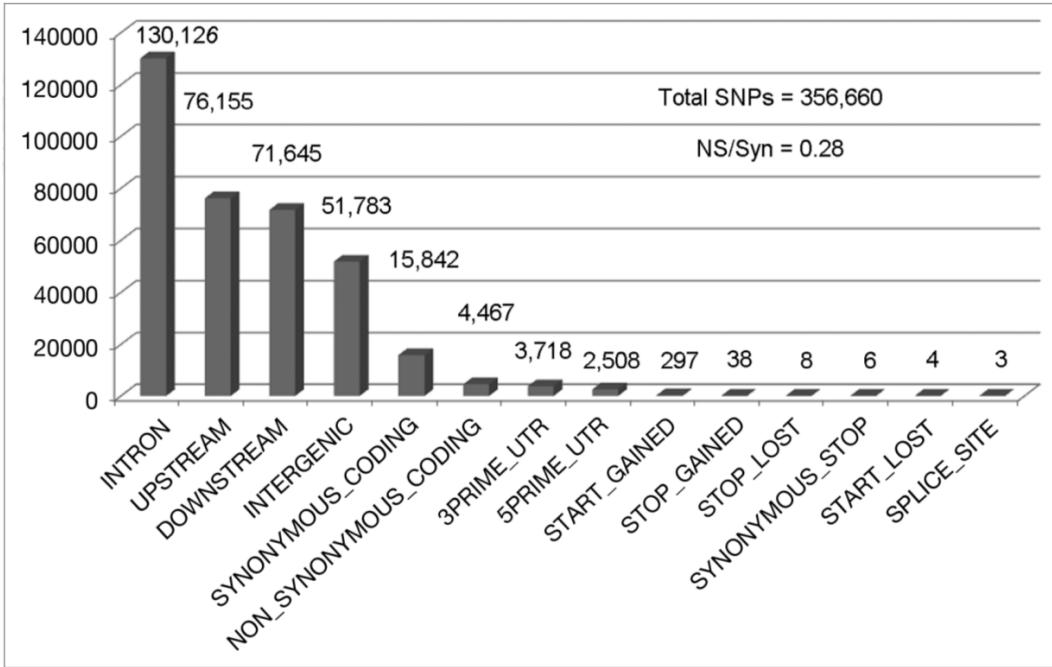


Figure 3.5: Classification of SNPs in  $w^{1118}; iso - 2; iso - 3$ . The number of NSPs in each class is shown above the bar. The quality score was arbitrarily set at 70 and above for this graph.

bilaterally-symmetric organisms in evolution [120]. The two stop-gained SNPs are in the non-conserved C-terminal region of Oc/Otd, which is thought to have a transcriptional-regulatory function. Since both strains are viable, both oc/otd genes are apparently functional although they encode a protein with 489 amino acids in  $w^{1118}; iso - 2; iso - 3$ , and a protein with 543 amino acids in the reference genome (Table 3.7).

An example of a stop-lost gene in class c, where the C-terminus is not conserved even among the Drosophila genera, is CG13958 that encodes a protein of unknown function (Fig. 3.11). In  $w^{1118}; iso - 2; iso - 3$ , CG13958 encodes a protein of 48 amino acids but in the reference genome it encodes a protein with 84 amino acids. When BLASTp was done with the non-redundant (nr) data set, there was not much homology beyond the 38<sup>th</sup> amino acid within the Drosophila genus. However, there was a near perfect (37/38) identity of the first 38 amino acids in four other Drosophila species: Drosophila grimshawi, Drosophila yakuba, Drosophila erecta and Drosophila virilis (Fig.3.11). This protein likely arose in the

Gene_name	bases from TSS	Gene_name	bases from TSS	Gene_name	bases from TSS
a	386 (-)	CG4766	367 (-)	MESR3	454 (-)
Ace	652 (-)	CG4839	293 (-)	Mipp2	67 (-)
Axn	107 (-)	CG5103 (2)	104/17 (-/-)	osp	358 (-)
btsz	228 (+)	CG6024	269 (-)	p120ctn	119 (-)
Calx	582 (+)	CG7985	60 (+)	Pld	144 (+)
CAP	1224 (+)	CG8026	612 (+)	Pli	196 (-)
CG10186	402 (+)	CG8176	128 (-)	Pvr (2)	472/915 (-/+)
sesn	147 (+)	cpo	168 (+)	pxb (2)	50/76 (-/-)
CG12355	151 (-)	dac	103 (-)	rib	2 (-)
CG13802 (3)	490/575/635 (-/-)	dpr15	433 (-)	rn	142 (-)
haf	89 (-)	EcR	160 (-)	Samuel	517 (-)
CG15086	114	Eip63E	171 (+)	sli	307 (-)
CG15878	52 (-)	fdl (2)	307/437 (-/-)	so	5252 (-)
CG18522	40 (-)	frtz	196 (-)	Sobp	24 (+)
CG30419	253 (-)	GC	76 (-)	sprt	358 (-)
CG31163	998 (-)	Gug	70 (-)	Strn-Mlck (2)	210/228 (+/+)
Dscam3	269 (-)	inv	771 (+)	tai	203 (-)
CG31688	430 (-)	lpk1	376 (-)	vn	1793 (-)
CG32048	63 (+)	klu	576 (+)	wg	231
CG32150	747 (+)	Mbs	10 (-)	Wnt4	680 (-)

Table 3.6: 60 Genes with start-gaineded SNPs with ATGs. Bases from TSS, bases from translation start site not including the ATG start-gained SNP. (+), in same reading frame as annotated ATG. (-), in different reading frame as annotated ATG.

Term	Count	%	pvalue	List Total	Pop Hits	Pop Total	Fold Enrichment	Bonferroni	Benjamini	FDR
tissue morphogenesis	21	8.898305	2.07E-08	147	247	7937	4.590515	2.37E-05	2.37E-05	3.33E-05
immunoglobulin-like	16	6.779661	3.40E-08	198	132	10196	6.241812	1.42E-05	7.08E-06	4.77E-05
developmental protein	29	12.28814	2.75E-07	229	540	12980	3.043992	3.99E-05	3.99E-05	3.27E-04
alternative splicing	31	13.13559	3.82E-07	229	616	12980	2.852464	5.53E-05	2.77E-05	4.53E-04
tissue morphogenesis	FRTZ, NRX-IV, ESG, WG, PBL, SFL, MBS, RIB, TOW, WNT4, FORM3, SLI, EIP63E, PHL, YRT, FAS, SRC64B, TWI, DLG1, BTSZ, HS6ST									
immunoglublin-ilke	CG31814, DPR15, PVR, DPR16, CG14521, KLG, VN, CG12484, BEAT-IB, CG10186, DPR2, STRN-MLCK, CG34371, KEK5, FAS, CG15630									
developmental protein	VN, ESG, DEI, INV, DAB, AWH, SCRIB, BICC, MST87F, WNT4, RIG, SLI, NUMB, PIP, INE, TWI, DLG1, FOXO, PTP10D, WG, AXN, EIP74EF, BUN, SO, FZ2, FDL, SCYL, SRC64B, POXN									
alternative splicing	CPO, CPN, ECR, VN, CG11299, RN, DAB, AWH, SCRIB, INX7, SLI, PIP, NRV2, INE, DLG1, L(1)G0196, CG32048, FOXO, PTP10D, CYCT, WG, EIP74EF, BUN, CG13624, GLUT1, OSP, FDL, SSP4, PHL, SCYL, RDGC									

Table 3.7: Genes with start-gained SNP GO categories in  $w^{1118}$ ;  $iso - 2$ ;  $iso - 3$ . Results of Gene ontology analysis for 297 start-gained SNPs in  $w^{1118}$ ;  $iso - 2$ ;  $iso - 3$ . Bottom, the genes in the indicated gene ontology category is listed.

Drosophila genus since it has no known homologs outside of this genus.

There are also five stop-lost SNPs in *w<sup>1118</sup>; iso-2; iso-3* (Table 3.7). All of these SNPs are in predicted protein-coding genes, metabotropic GABA-B receptor subtype 1 (GABA-B-R1), CG13958, CG4975, brown (bw), and POU domain motif 3 (pdm3). It is not known whether any of the these genes are essential in Drosophila besides bw, which is not required for viability. However, the metabotropic GABA-B receptor subtype 1 (GABAB-R1) gene is required for normal behavior in mice [120] and the ortholog is therefore likely also essential in Drosophila, although no phenotypic data are available ([www.flybase.org](http://www.flybase.org)). The bw gene is classic gene first described in 1921 by Waaler, [236] which causes the eyes to be brown rather than red and encodes an ATPase binding cassette (ABC) transporter [207]. The bw 1 mutation in the reference strain is a spontaneous allele with a 412-transposon repeat insertion [72], which would have been missed in our nextgeneration sequencing data because the input sequence we analyzed contained only short-read sequences that mapped uniquely to the reference genome.

Not much is known about the functions of several genes with in-frame stop-gained SNPs. The pdm3 gene is expressed in the larval and adult nervous system, and it encodes a highly-conserved Hox domain, but no phenotypic data are available ([www.flybase.org](http://www.flybase.org)). No phenotypic data are available for either CG13958 or CG4975. The protein encoding CG13958 has no known conserved domain, and its peak expression is observed within 0624 h of embryogenesis, during early larval stages, at stages throughout the pupal period, and in the adult male ([www.flybase.org](http://www.flybase.org)). The protein encoded by CG4975 has an Armadillolike helical domain and an Ataxin-10 domain and has expression in the hind gut during the late larval and periods ([www.flybase.org](http://www.flybase.org)) [36].

Some of the stop-lost SNPs have interesting consequences. For example, a stop-lost SNP in *w<sup>1118</sup>; iso-2; iso-3* is in the CG13958 gene and causes an extension of eight amino acids before the next stop codon in 3' UTR sequence is reached (Fig. 3.13). Since the C-termini of CG13958 vary in *w<sup>1118</sup>; iso-2; iso-3* and the reference strains of Drosophila melanogaster,

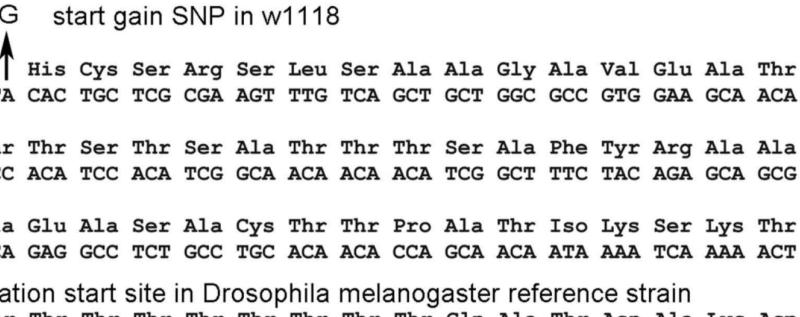
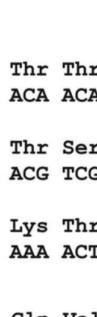
<b>A</b> G start gain SNP in <i>w<sup>1118</sup></i>  Stop His Cys Ser Arg Ser Leu Ser Ala Ala Gly Ala Val Glu Ala Thr Thr Thr TGA CTA ATA CAC TGC TCG CGA AGT TTG TCA GCT GCT GCC GCG GTG GAA GCA ACA ACA ACA  Lys Leu Thr Thr Ser Thr Ser Ala Thr Thr Ser Ala Phe Tyr Arg Ala Ala Thr Ser AAA CTA ACC ACA TCC ACA TCG GCA ACA ACA TCG GCT TTC TAC AGA GCA GCG ACG TCG  Ala Ser Ala Glu Ala Ser Ala Cys Thr Thr Pro Ala Thr Iso Lys Ser Lys Thr Lys Thr GCG TCG GCA GAG GCC TCT GCC TGC ACA ACA CCA GCA ACA ATA AAA TCA AAA ACT AAA ACT  → translation start site in <i>Drosophila melanogaster</i> reference strain Met Ala Thr Thr Thr Thr Thr Thr Gln Ala Thr Asn Ala Lys Asp Gln Val ATG GCC ACC ACC ACA ACA ACA ACG GGG GCA ACA AAT GCT AAA GAT GGC GTC	<b>B</b>  <pre>           D mel 1      MHCSRSLSAAGAVEATTKLTTSTSATTSA-FYRAATSASAEASACTTPATIKSKTKTMA 60                       MHCSRSLSAAGAV+ATTT      T+++ TT++ FYRAATSASAEAS CTT           D yak 1      MHCSRSLSAAGAVDATTTLKLTTSATTSAFYRAATSASAEAVCTT-PATTKSKTAKM 61           D mel 61      TTTTTTTTGATNAKDGVTMREKKGGALQKLKKRILSHSFGRLTISREDGDESTHHHHHHH 120                       TTTTTTTGATNAKDGVTMREKKGGALQKLKKRILSHSFGRLTISREDGDESTHHHHHHH           D yak 62      ATTTTTTTGATNAKDGVTMREKKGGALQKLKKRILSHSFGRLTISREDGDESTHHHHHHH 121         </pre>
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Figure 3.8: Analysis of Eip63E start-gained SNP in *w<sup>1118</sup>; iso – 2; iso – 3*. (A), Location of the start-gained SNP at the Eip63E locus. Notice that the reading frame is the same as the normal translation start site (TSS). (B), Conservation of 60 amino acid N-terminal region of Eip63E in *w<sup>1118</sup>; iso – 2; iso – 3* with *Drosophila yakuba* orthologous gene. The other sequenced *Drosophila* species do not have this N-terminal sequence (not shown).

<b>stop gained</b>	<b>location</b>	<b>length</b>	<b>phenotype</b>	<b>stop gained</b>	<b>location</b>	<b>length</b>	<b>phenotype</b>
ade3	255K/*	435	ND <sup>a</sup>	ex	693Q/*	1428	Lethal <sup>d</sup>
CG10126	11W/*	228	ND <sup>a</sup>	Ibk	1130Y/*	1174	ND <sup>d</sup>
CG15394	120Q/*	186	ND <sup>a</sup>	MESR4	1509E/*	2072	lethal <sup>d</sup>
CG31145	27L/*	764	ND <sup>a</sup>	mew	752Q/*	1050	Lethal <sup>a</sup>
CG31784	1049Q/*	1078	ND <sup>a</sup>	NFAT	12G/*	1420	ND <sup>d</sup>
CG32115	468W/*	476	ND <sup>a</sup>	oc/otd	389Y/*, 453Y/*	543	Lethal <sup>d</sup>
LRP1	2917Y/*, 2918E/*	4700	ND <sup>a</sup>	Pde9	255C/*	1527	ND <sup>a</sup>
CG34006	121R/*	202	ND <sup>b</sup>	rho-4	140W/*	418	ND <sup>a</sup>
CG34326	49Y/*	84	ND <sup>c</sup>	Synd	375S/*	495	ND <sup>a</sup>
CG3493	1419E/*	1490	ND <sup>a</sup>	tai	1420Q/*	2048	Lethal <sup>d</sup>
CG3964	509Y/*	983	ND <sup>a</sup>	trol	811Y/*, 808E/*	4180	Lethal <sup>a</sup>
CG4068	379Q/*	623	ND <sup>d</sup>	<b>stop lost</b>			
CG7236	70E/*	502	ND <sup>a</sup>	GABA-B-R1	*/L (+9 aa)	837	ND <sup>a</sup>
Cht6	4175L/*	4542	ND <sup>a</sup>	CG13958	*/G (+8 aa)	539	ND <sup>a</sup>
Cyp4s3	260W/*	496	ND <sup>a</sup>	CG4975	*/Q (+1aa)	353	ND <sup>a</sup>
Dif	263C/*	668	lethal <sup>a</sup>	bw	*/Q (+71 aa)	417	eye color <sup>c</sup>
Dp	17353L/*	22972	Lethal <sup>a</sup>	CG14755/pdm3	*/Q (+5 aa)	285	ND <sup>a</sup>

Table 3.9: Stop gained and stop lost in *w<sup>1118</sup>; iso - 2; iso - 3*. Stop gained, gene with stop gained SNP. Location, amino acid number changed to a stop codon (e.g., 255K/\*, indicates lysine at amino acid changed to a stop codon). Length, the length of the protein in amino acids. Phenotype, not determined (ND), withdrawn (no longer considered a gene by FlyBase), and NPC (non-protein coding, such as a rRNA). For stop lost SNPs (bottom), \*/L (+9 aa) indicates that the next in frame stop is after nine additional amino acids are added. a-d refer to SNP categories 14 (see text).

it is conceivable that the C-terminus might also fluctuate in other *Drosophila* species. To test this idea, we investigated the C-terminal regions of CG13958 homologs in other *Drosophila* species.

We found that CG13958 homologs have variable C-terminal amino acids in different species of *Drosophila*. When the CG13958 protein is analyzed by protein Basic Local Alignment Search Tool (BLASTp) with the non-redundant (nr) protein database (<http://www.ncbi.nlm.nih.gov/>), at least two *Drosophila* species have extended C-terminal amino acids and at least three *Drosophila* species have missing amino acids at the C-termini (Fig. 3.13). For example, *Drosophila pseudoobscura* has three of the extended amino acids found in *w<sup>1118</sup>; iso - 2; iso - 3* and *Drosophila mojavensis* has four of them. In contrast, *Drosophila simulans* is missing the last terminal amino acid, *Drosophila erecta* is missing the last two terminal amino acids, and *Drosophila yakuba* is missing the last three amino acids found in the reference strain (Fig. 3.13). The large number of stop-gain and stop-lost SNPs in *Drosophila*

likely has important implications on the evolution of protein function (see Discussion).

Synonymous and non-synonymous SNPs in *w*<sup>1118</sup>; *iso* – 2; *iso* – 3. There are 15,842 synonymous SNPs and 4,467 nonsynonymous SNPs in annotated coding regions in *w*<sup>1118</sup>; *iso* – 2; *iso* – 3 (Fig. 3.5). A synonymous SNP (silent SNP) is defined as a SNP that does not change the amino acid in the protein, whereas a nonsynonymous SNP does. The genome-wide normalized *N/S* ratio (*dN/dS*), also called  $\omega$  (i.e.,  $\omega = dN/dS$ ), is by definition normalized to 1 in most evolutionary studies [222]. The non-normalized *N/S* ratio is  $\tilde{0.28}$  in *w*<sup>1118</sup>; *iso* – 2; *iso* – 3 compared with the reference genome, *y*<sup>1</sup>; *cn*<sup>1</sup>*bw*<sup>1</sup>*sp*<sup>1</sup> (i.e., *N/S* = 4,467/15,842; Table 3.1).

We examined the distribution of synonymous and nonsynonymous SNPs genome-wide for *w*<sup>1118</sup>; *iso* – 2; *iso* – 3 and saw higher levels of both classes of SNPs in the middle of the chromosome arms and lower levels near the centromeres and telomeres (Fig. 3.12 and left). This was expected because the number of SNPs is proportional to the recombination frequencies in the different regions of the chromosomes [17, 35]. Also, our previous analyses of the distribution of total SNPs revealed a similar pattern. We observed higher *N/S* ratios near the telomeres and centromeres and lower *N/S* ratios in the middle of the chromosome arms (Fig. 3.12 and right).

## 3.5 Discussion

In this paper, we used SnpEff to categorize the  $\sim 356,660$  SNPs in *w*<sup>1118</sup>; *iso* – 2; *iso* – 3 and place them into 14 different classes based on their predicted effects on protein function. In order of prevalence, these 14 classes are intron, upstream, downstream, intergenic, synonymous, non-synonymous, 3' UTR, 5' UTR, start-gained, stop-gained, stop-lost, synonymous-stop, start-lost and splice-site SNPs (Fig. 3.5). The reason for cataloging the SNPs in *w*<sup>1118</sup>; *iso* – 2; *iso* – 3 is to get a better appreciation of evolution of genome sequences and genome organization in this common laboratory strain. We appreciate the fact that both *w*<sup>1118</sup>; *iso* – 2; *iso* – 3

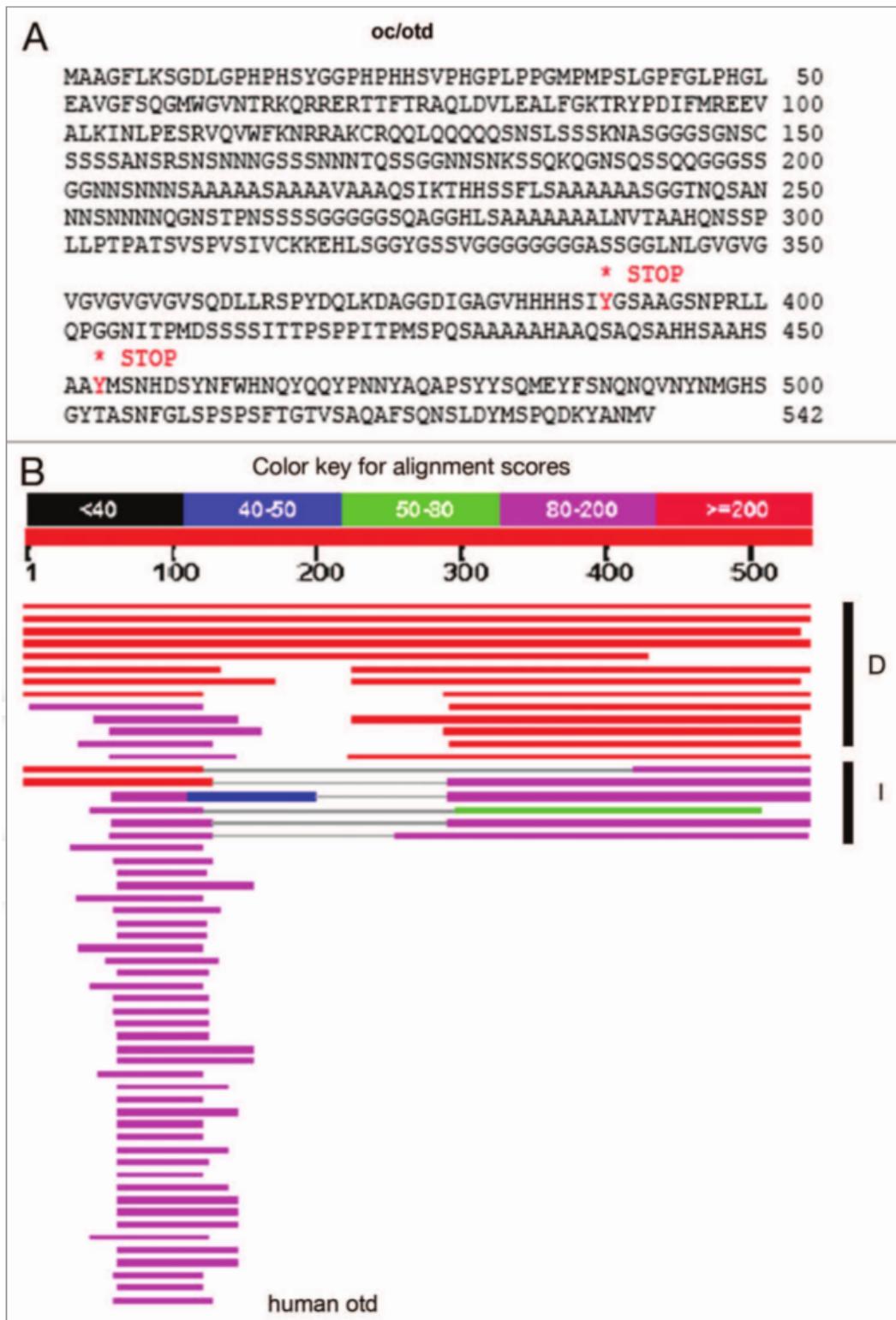


Figure 3.10: Oc/Otd has two stop-gained SNPs in *w<sup>1118</sup>*; *iso-2*; *iso-3*. (A) Location of the two stop gained SNPs in oc/otd. (B) Protein BLAST of Oc/Otd against the non-redundant (nr) protein database shows that only the 60 amino Hox domain flanking amino acid 100 is conserved from Drosophila to humans. The color coding shows the alignment scores.

and  $y^1; cn^1bw^1sp^1$  are derived and highly manipulated laboratory strains and do not represent natural populations. Therefore, we do not mean to imply that the analyses in this paper are significant but rather just observational. To be meaningful, these observations need to be followed up with natural populations. Hundreds of Drosophila natural populations have already been or are in the process of being sequenced, so this should be feasible in the near future with a program such as SnpEff [10].

Many of the stop-gained and stoplost SNPs in  $w^{1118}; iso - 2; iso - 3$  occur in essential genes that apparently still function after amino acid truncations caused by the stop-gained SNPs (Table 3.7). These non-critical effects of the stop-gained SNPs are worth noting because nonsense codons in the transcribed mRNAs generally result in nonfunctional protein products. For example, some genetic disorders, such as thalassemia and Duchenne muscular dystrophy (DMD), result from nonsense SNPs [84, 229, 34]. Also, nonsense SNP-mediated RNA decay exists in yeast, Drosophila and humans, and usually ensures that mRNAs with premature stop codons are degraded [88].

The stop-gained and stop-lost SNPs in essential genes, if they are validated, could have profound evolutionary implications and suggest the involvement of prions, analogous to  $[PSI^+]$ , in the retention and selection of these SNPs. Brian Cox, a geneticist working with the yeast *Saccharomyces cerevisiae*, discovered  $[PSI^+]$  in 1965 as a non-genetically transmissible trait with a cytoplasmic pattern of inheritance similar to mitochondria [57]. He isolated a yeast strain auxotrophic for adenine due to a nonsense mutation is able to survive in media lacking adenine when  $[PSI^+]$  is present [57]. Reed Wickner showed in 1994 that  $[PSI^+]$  resulted from a prion form of the translation termination factor, Sup35 [246]. Lindquist and colleagues showed in 2008 that the  $[PSI^+]$  prion provides survival advantages in several stressful environments, such as high salt conditions [232]. They have speculated that Sup35 is an evolutionary capacitor that, when inactivated in the  $PSI^+$  form, releases cryptic genetic variation that allow expression of novel C-terminal amino acids in hundreds of proteins, some of which are beneficial in stressful environments.

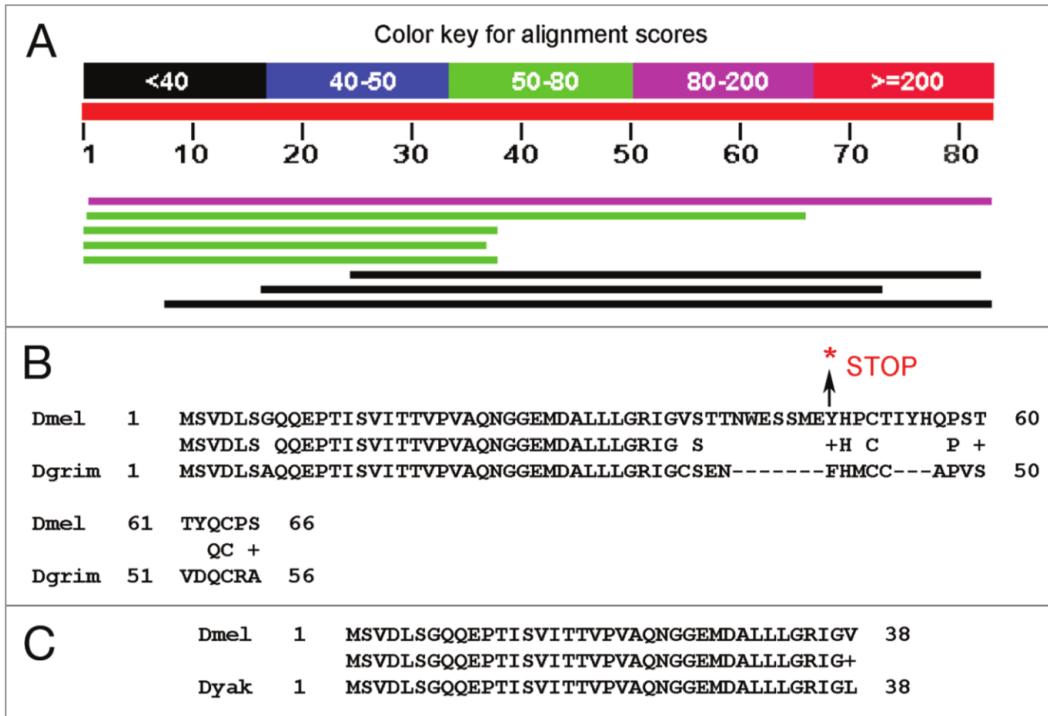


Figure 3.11: CG34326 has one stop-gained SNP in *w<sup>1118</sup>*; *iso-2*; *iso-3* in the non-conserved C-terminal region. (A) Protein BLAST of CG34326 against the non-redundant (nr) protein database shows that only the 38 N-terminal amino acids are conserved among Drosophila species and not beyond Drosophila. The colored lines represent the homologs from the following organisms: Drosophila melanogaster, Drosophila grimshawi, Drosophila yakuba, Drosophila erecta, Drosophila virilis, Ixodes scapularis, Ixodes scapularis and Nycticebus coucang. (B) Alignment of Drosophila melanogaster CG34326 with orthologous gene from Drosophila grimshawi. (C) Alignment of Drosophila melanogaster CG34326 with orthologous gene from Drosophila yakuba.

How might prions be involved in revealing cryptic genetic variation in the 5' and 3' UTRs? While most prions are thought to not directly mutate DNA sequences, they could provide an environment that would make the retention and selection of beneficial SNPs more likely. For example, a stop-lost SNP would allow a modified protein with the new C-terminal tail to be always expressed, even when the prion is lost [232]. Therefore, a stop-lost SNP would more likely occur in a strain with beneficial codons in the 3' UTR because the cryptic C-terminal amino acids encoded by these nucleotides would provide a selective advantage in stressful (i.e.,  $[PSI^+]$ ) environments when they are translated.

It is attractive to speculate that a similar prion-mediated evolutionary mechanism might

occur in Drosophila, for both stoploss and stop-gained SNPs, and that this might help explain the large number of SNPs that we see in these categories. We note that Drosophila has several Sup35 orthologs, some of which have N-terminal repeats that are known to be potentially prion-forming domains [232]. We acknowledge that this is a highly speculative explanation for the high numbers of start-gained and stop-lost SNPs, but we believe that it is worthy of further investigation.

The many potential start-gained SNPs in Drosophila might also have evolutionary implications. Similar to the cryptic genetic variation that is revealed by stop-lost mutations in the 3' UTR, start-gained SNPs reveal cryptic genetic variation in the 5' UTR. Uncovering the cryptic genetic variation in times of environmental stress, such as by inducing transcription initiation at start sites upstream of the normally-used transcription start sites, could be one mechanism to facilitate the use of potential start-gained SNPs. Further mutations and selection of the potential start-gained SNPs, such as by introducing better Kozak consensus sequences or more commonly used 5'-AUG-3' translation initiation codons, can stabilize the cryptic genetic variation further if it leads to improved survival or reproductive fitness in a stressful environment. While amino acid extensions and deletions in known essential genes occur only 8 times in *w<sup>1118</sup>; iso - 2; iso - 3* compared with the reference strain (Table 3.9), as laboratories begin to sequence hundreds or even thousands of individuals in a population, extensions and deletions are likely to be found in a large proportion of functional genes.

Finally, we recently upgraded SnpEff further by including over 320 databases for different reference genome versions that can be analyzed (<http://snpeff.sourceforge.net/SnpSift.html>). Sources of information for creating these databases are ENSEMBL, UCSC Genome Bioinformatics website as well as organism specific databases, such as FlyBase (*Drosophila melanogaster*), WormBase (*C. elegans*) and TAIR (*Arabidopsis thaliana*), to name a few. The program SnpEff is open access and additional genomes can be added and assistance in using SnpEff can be provided upon request. Rapid analyses of whole-genome sequencing data should now be feasible to perform by any laboratory

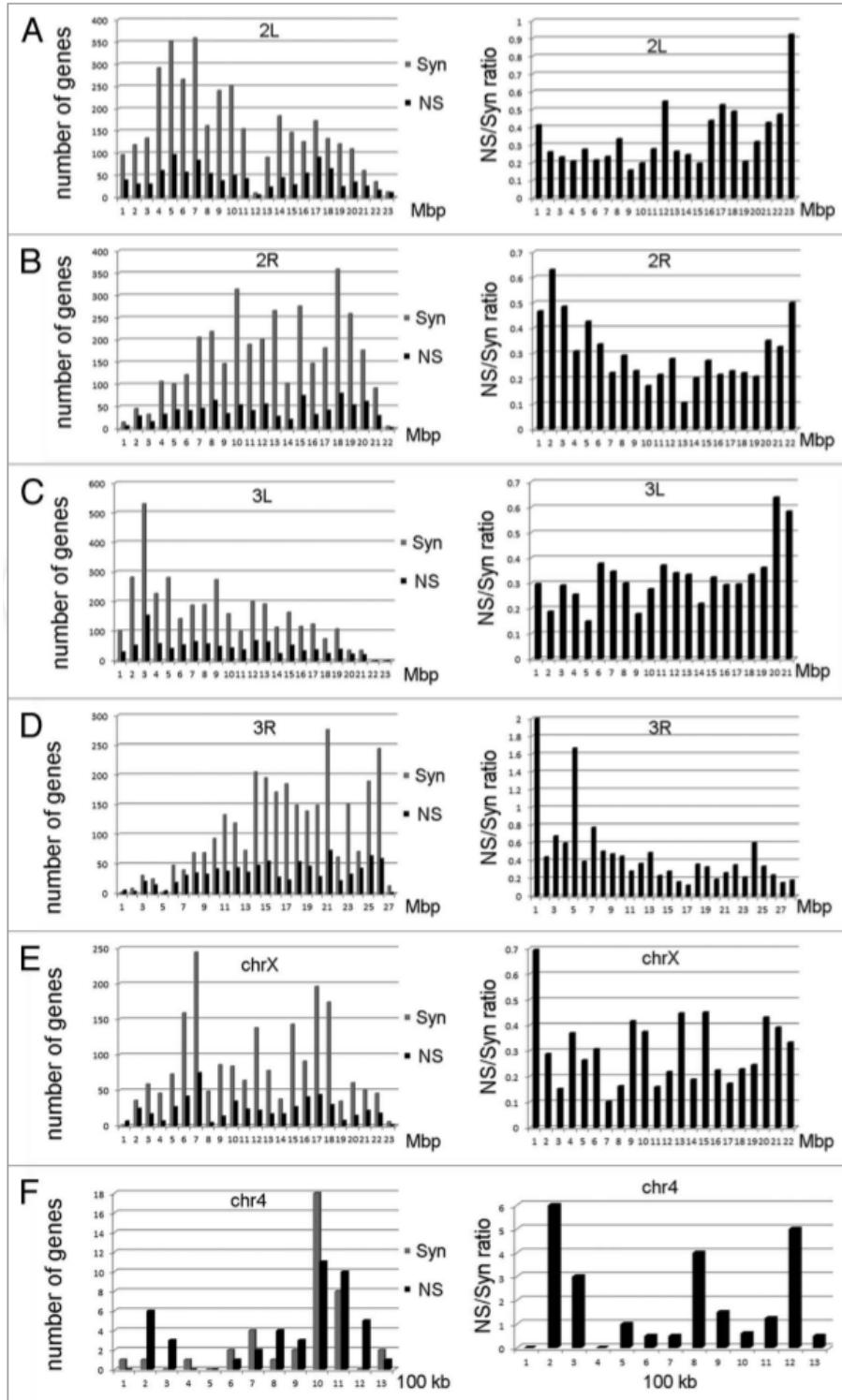


Figure 3.12: Nonsynonymous to synonymous ratios along the chromosome arms in  $w^{1118}; iso - 2; iso - 3$ . (A) Left, Nonsynonymous SNPs at 1 Mbp intervals along the 2L chromosome arm (black) and synonymous SNPs (gray). Right,  $N/S$  ratios (NS/Syn) along the chromosome arms. Notice that  $N/S$  ratios are higher near the centromere and telomere (see text). (BF) as in (A), but for chromosome arms 2R, 3L, 3R, 4 and X.

## 3.6 Methods

**SnpEff overview.** The program is divided in two main parts (i) database build and (ii) effect calculation. Part (i) Database build is usually not run by the user, because many databases containing genomic annotations are available. Databases are build using a reference genome, a FASTA file, and an annotation file, usually GTF, GFF or RefSeq table, provided by ENSEMBL, UCSC Genome Bioinformatics website or other specific websites, such as FlyBase, WormBase and TAIR. SnpEff databases are gzip serialized objects that represent genomic annotations.

Part (ii) Effect calculations can be performed once the user has downloaded, or built, the database. The program loads the binary database and builds a data structure called “interval forest,” used to perform an efficient interval search (see next section). Input files, usually in VCF format, are parsed and each variant queries the data structures to find intersecting genomic annotations. All intersecting genomic regions are reported and whenever these regions include an exon, the coding effect of the variant is calculated (hence the name of the program). A list of the reported effects and annotations is shown in Table 3.2, additional information produced by the program, is shown in Table 3.3 and Table 3.4, for different output formats.

**SnpEff algorithms.** In order to be able to process thousands of variants per second, we implemented an efficient data structure that allows for arbitrary interval overlaps. We created an interval forest, which is a hash of interval trees indexed by chromosome. Each interval tree [54] is composed of nodes. Each node has five elements (i) a center point, (ii) a pointer to a node having all intervals to the left of the center, (iii) a pointer to a node having all intervals to the right of the center, (iv) all intervals overlapping the center point sorted by start position and (v) all intervals overlapping the center point, sorted by end position.

Querying an interval tree requires  $O(\log n + m)$  time, where  $n$  is the number of intervals in the tree and  $m$  is the number of intervals in the result. Having a hash of trees, optimizes the search by reducing the number of intervals per tree.

In order to create this the interval forest, genomic information can be parsed from three main annotation formats: GTF (version 2.2), GFF (versions 3 and 2), UCSC Genome Bioinformatics website RefSeqTables and tab separated text files (TXT). Once the interval forest is created, the structure is serialized and compressed (GZIP) into a binary database. There are over 250 genomic binary databases that are currently distributed with SnpEff, which include all genomes from ENSEMBL.

**SnpEff accuracy.** As part of our standard development cycle, we perform accuracy testing by comparing SnpEff to ENSEMBL “Variant effect predictor,” which we consider it is the “gold standard.” Current unity testing includes over a hundred test cases with thousands of variants each to ensure predictions are accurate.

**SnpEff integration.** SnpEff provides integration with third party tools, such as Galaxy [89], which creates a web based interface for bioinformatic analysis pipelines. Integration with Genome analysis tool kit 4 (GATK) was provided by the GATK team. Detailed information on how to download, install and run, as well as usage examples of the program, can be found at <http://snpEff.sourceforge.net>.

Data access. SnpEff Data can be accessed from the Supplemental data file for *w<sup>1118</sup>; iso – 2; iso – 3* or by contacting D.M.R.

Disclosure of Potential Conflicts of Interest No potential conflicts of interest were disclosed.

### 3.7 Acknowledgements

This work was supported by a Michigan Core Technology grant from the State of Michigan's 21<sup>st</sup> Century Fund Program to the Wayne State University Applied Genomics Technology Center. This work was also supported by the Environmental Health Sciences Center in Molecular and Cellular Toxicology with Human Applications Grant P30 ES06639 at Wayne State University, NIH R01 grants (ES012933) to D.M.R. and DK071073 to X.L. We thank

**Drosophila melanogaster**

Dm-ref	522	LVLQQCDSVQGYMEVSL*	538	
		LVLQQCDSVQGYMEVSL*		+8
Dm-w <sup>1118</sup>	522	LVLQQCDSVQGYMEVSLQIFNNINI*	546	

**Drosophila simulans**

Dm-ref	522	LVLQQCDSVQGYMEVS	537	
		LVLQQCDSVQGYMEVS		-1
Sbjct	522	LVLQQCDSVQGYMEVS	537	

**Drosophila erecta**

Dm-ref	522	LVLQQCDSVQGYMEV	536	
		LVLQQCDSVQGYMEV		-2
Sbjct	522	LVLQQCDSVQGYMEV	536	

**Drosophila yakuba**

Dm-ref	481	LVLQQCDSVQGYME	535	
		LVLQQCDSVQGYME		-3
Sbjct	481	LVLQQCDSVQGYME	535	

**Drosophila mojavensis**

Query	522	LVLQQCDSVQGYMEVS-LQIF	541	
		LVLQQCDSVQGY+EV L+IF		+3
Sbjct	517	LVLQQCDSVQGYIEVRYLKIF	537	

**Drosophila pseudoobscura pseudoobscura**

Query	522	LVLQQCDSVQGYMEVSLQIFN	542	
		LVLQQCDSVQGY+EV +F+		+4
Sbjct	571	LVLQQCDSVQGYIEVFCALFH	591	

Figure 3.13: CG13958 has a stop lost SNP in *w<sup>1118</sup>; iso-2; iso-3*. The top comparison shows the alignment of the Drosophila melanogaster reference genome with *w<sup>1118</sup>; iso-2; iso-3*. Notice that the stop lost causes an extension of 9 amino acids. The second through sixth comparisons shows the alignment of Drosophila simulans, Drosophila erecta, Drosophila yakuba, Drosophila mojavensis and Drosophila pseudoobscura pseudoobscura (Sbjct) with the Drosophila melanogaster reference genome (Dm-ref). The number of terminal amino acids missing or gained is shown (-1 to +3).

David Roazen, Eric Banks and Mark DePristo in the GATK team at the Broad Institute who integrated SnpEff with the Genome Analysis Toolkit (GATK).

Note Supplemental material can be found at: <http://www.landesbioscience.com/journals/fly/article/19695>

## 3.8 Epilogue

At the beginning of my Ph.D., functional annotation of genomic variants was an unsolved problem with many research labs creating in-house custom solutions that oftentimes were inefficient and lacking of rigorous testing. As a consequence, shortly after SnpEff & SnpSift were released they quickly became widely adopted by the research community as well as many private organizations. Currently SnpEff & SnpSift has over 250 downloads per week (as reported by SourceForge, where the tools are hosted). So far SnpEff & SnpSift have been cited over 400 times.

### 3.8.1 Data structures for annotations

A very simple approach used by ANNOVAR [238] is to create an index by dividing each chromosome into  $N$  bins of equal size. All genomic features are stored in a hash table indexed by chromosome name and bin number. This approach has running time of  $O(n)$  where  $n$  is the number of features, but it can be easily tuned by creating small bins, at the cost of increased memory requirements.

Another approach [39] is to use an “interval forest”, which is a hash of “interval trees” indexed by chromosome. Each interval tree is composed of nodes. Each node has five elements i) a center point, ii) a pointer to a node having all intervals to the left of the center, iii) a pointer to a node having all intervals to the right of the center, iv) all intervals overlapping the center point sorted by start position, and v) all interval overlapping the center point sorted by end position. Querying an interval tree requires  $O[\log(n) + m]$  time,

where  $n$  is the number of features in the tree and  $m$  is the number of features in the result. Having a hash of trees optimizes the search by reducing the number of intervals per tree.

# Chapter 4

## Epistatic GWAS analysis

### 4.1 Preface

In recent years over 80 genetic loci related to type II diabetes (T2D) have been identified [165, 48]. However the combined effect of all these loci account for less than 10% of the overall disease predisposition [155]. This poses the question of why, given that so much effort has been directed at finding the genetic components of this disease, the loci found so far have such modest effects. The lack of large genetic effects is known as the “missing heritability” problem and does not only arise in T2D but also in almost all complex traits. Recent studies on the topic of missing heritability [261, 262] suggest that genetic interactions (epistasis) might be at least partly responsible for this issue.

In this chapter, we propose a novel framework that examines the potential for putative epistatic interactions in the context of genome wide association studies (GWAS) to uncover potentially interacting variants that might affect disease risk. Although the computational approach we describe is applicable to any complex trait, in this chapter we apply it to diabetes GWAS data. Type II diabetes (T2D) is a complex disease that was first described by the Egyptians in 1500 BCE. Later the Greeks in 230 BCE used the term “diabetes” meaning “pass through” (or “siphon”) denoting the constant thirst and frequent urination

of the patients. In the 1700s the term “mellitus” (from honey) was added to denote that the urine was sweet and would “attract ants”.

Diabetes symptoms include frequent urination, thirst, and constant hunger, high blood sugar (hyperglycemia) and insulin resistance. Long term complications from T2D may include eyesight problems, heart disease, strokes and kidney failure. Type II diabetes is highly correlated with obesity and the disease rate has increased dramatically during the last 50 years. According to the World Health Organisation the prevalence of diabetes is around 8% to 9% in adults and an estimated 1.5 million deaths were caused by diabetes in 2012 [100], which is predicted to be the 7th leading cause of death by 2030. The costs associated to treating diabetes patients in the U.S. alone are estimated around \$245 billion dollars.

The rest of the chapter is from the following paper: **P. Cingolani**, R. Sladek, M. Blanchette, “A co-evolutionary approach for detecting epistatic interactions in genome-wide association studies”, to be submitted to PLOS Computational Biology.

## 4.2 Abstract

**Motivation** Epistasis, broadly defined as genetic interactions, is one of the likely factors explaining why variants identified to date by genome-wide association studies (GWAS) account for a small portion of heritable risk in complex diseases. Due to their high complexity, reduced statistical power and sometimes prohibitive computational requirements, epistatic GWAS analyses have rarely been performed.

**Methods** In this paper, we propose a novel methodology for identifying putative epistatic interactions by combining interspecies comparison and population level variation. Using crystal structures for individual proteins and protein complexes as well as genome wide multiple species alignment, we create a co-evolutionary substitution model that allows the calculation of the posterior probability of physical interaction between residues. These probabilities are then used as the interaction priors for an epistatic GWAS analysis using a

Bayesian framework.

**Results** Our algorithms can be applied to genome scale sequencing studies for tens of thousands of samples, that typically yield millions of variants. We applied our approach to a large type II diabetes (T2D) case-control cohort and inferred a number of putative interactions associated with increased risk of developing T2D.

**Availability** Our code is publicly available at [github.com/pcingola/Epistasis](https://github.com/pcingola/Epistasis)

### 4.3 Introduction

Genetic studies aim to discover how a phenotype of interest, such as disease risk or height, is affected by an individual's genetic background. Genome wide association studies (GWAS) are powerful techniques aimed at finding statistical associations between a phenotype and genetic variants [44]. Nevertheless, variants discovered in GWAS so far can only explain a small part for the phenotypic heritability of complex traits. For instance, all genetic variants associated with height collectively account for a few centimetres in the offspring's height [250]. Similarly the known variants related to type 2 diabetes risk collectively explain only 5% to 10% of the overall variance in disease predisposition [165, 48]. This problem is known as “missing heritability” [155] and recent theories suggest that genetic interactions (epistasis) might play an important role in it [261, 262].

The foundations for epistasis [86] were proposed almost a hundred years ago by Bateson (1909) and Fisher (1918). It was the latter who coined the term to denote a “statistical deviation of multi-locus genotype values from an additive linear model for the value of a phenotype” [86]. There is evidence of such interactions being involved in complex diseases. For instance an interaction between BACE1 and APOE4 having a significant association with Alzheimer's disease has consistently been replicated in different studies [46]. Many types of situations can lead to epistatic interactions, perhaps the most common involves

pairs of variants that encode amino acids whose physical interaction is required for their respective proteins to function in a pathway [53, 52].

One of the main problems in finding association between interactions and disease is that out of the whole set of molecular interactions (the interactome) only a small part has been characterized [234]. Interacting proteins can be identified experimentally through several types of approaches (yeast two hybrid, protein fragment complementation assay, glutathione-s-transferase, affinity purification coupled to mass spectrometry, tandem affinity purification, etc. [215]) and large databases of protein-protein interactions are now available [220, 215]. In almost all cases, these methods identify the presence of an interaction between proteins but do not discern the exact residues mediating the interaction. Furthermore, it is estimated that up to 80% of human protein-protein interactions remains unknown [234].

These issues can be partially addressed using computational predictions of either pairs of interacting proteins or interacting residues [216]. A type of approach that has been gaining popularity recently is one that makes use of the plethora of genomic sequences available for species other than human in order to discover evolutionary evidence of selective pressure on pairs of residues to identify interacting sites and interfaces [156]. Interacting residues and their neighbours may then be subject to compensating epistasis, where the effect of a mutation at a residue in one protein may be offset by another mutation at a residue in the second protein [178]. This is a phenomenon generically known as co-evolution. For example assuming that evolutionary pressure acts on both interaction sites simultaneously, co-occurring compensatory mutations can become fixed in the population with higher probability than non-compensatory ones. In light of this hypothesis, one can use statistical methods to analyse multiple sequence alignments of proteins from different organisms to find co-evolving sites. This approach has been used to identify co-evolving sites both within a protein (e.g. N-terminal and C-terminal domains in PKG protein [94], GroES-L chaperoning system [202],  $\alpha$  and  $\beta$  haemoglobin subunits [178]), and between interacting proteins (e.g. G-protein coupled receptors and protein ligands [94]).

Many methods exist to find putative interacting loci, both within and across proteins, based on evolutionary evidence (see [67] for a review). The earliest methods for inferring co-evolution used either correlation or mutual information between two loci [156] in a multiple sequence alignment. However, these methods are known to have systematic biases due to the fact that they ignore phylogenetic relationships [67] or sequence heterogeneity problems [243]. More sophisticated methods, such as DCA [164], PSICOV [119] or mdMI [42] try to overcome these biases by using global statistical models, however they are not suitable for GWAS-scale analysis for two main reasons. First, they require multiple alignments of a very large number of sequences (ranging from 400 to  $25L$ , where  $L$  is the length of the protein [42]), and such depth remains only available for a small number of proteins. Second, they are computationally demanding (e.g. running for minutes or even days for each interacting pair of proteins being considered), making them unsuitable for analyses involving millions of variants spanning over thousands of proteins. Furthermore, a recent study shows that overall agreement between methods is not high (65% or less) and predictive power is quite low (only 6% of the “top scoring pairs” are real interactions) [42].

Considering epistatic interactions in GWAS is challenging for several reasons: i) interaction models are non-linear [86]; ii) analysing all order  $N$  variant combinations requires great computational power and efficient algorithms or might just be infeasible because the number tests grows exponentially with  $N$  [182]; iii) multiple hypothesis testing correction can render association tests underpowered for all but very large cohorts [86, 182]; iv) there is no consensus of what genetic interaction means, which is reflected in the difficulty to find a unified model [182, 154]; and v) the required sample sizes to detect epistatic interactions depend on phenotypic effect size and variants’ allele frequencies with some estimates assuming on the order of 10,000 to 500,000 cases [122] to be required (such cohorts are only now becoming feasible due to improvements and cost reductions in sequencing technology). For all these reasons the application of epistatic models to sequencing studies has not been widespread.

Approaches for epistatic GWAS do exist and they are based on a wide array of method-

ologies. In Zhao et al. [258], the authors infer epistatic interaction probabilities by noting that interactions create linkage disequilibrium patterns in the disease population. A Bayesian framework in Zhang et al. [257] takes into account several risk models and uses Dirichlet priors to solve each model analytically, then they combine them in the full model posterior distribution calculated using an MCMC sampling technique. In Ackermann et al. [2], the authors look for over / under-represented allele pairs in a given family pedigree by performing an analysis of imbalanced allele pair frequencies. Finally, finding interacting variants can be viewed as an attribute selection problem, thus many machine learning methodologies have been proposed [161]. While all algorithms have relative advantages, there is no standard in epistatic GWAS analysis and we believe that better methods can be created by combining other sources of biological information, such as evolutionary evidence.

In this work we propose an approach to prioritize pairs of variants identified in case/control cohorts by combining genome wide association with epistatic interaction models. In a nutshell, our method uses a 100-way vertebrate genome alignment [22] to calculate interaction posterior probabilities for any given pair of residues in human proteins. This is achieved by contrasting the likelihood of the observed pair of alignment columns under a joint substitution model that factors in dependencies between interacting sites, and a null model of independent evolution. The posterior probabilities can then used as priors to modulate the evidence of epistatic interaction derived from GWAS data. Our implementation is efficient enough to be applied to GWAS-scale datasets of tens of thousands of samples. Finally we showcase this method using a cohort of  $\sim 13,000$  individuals in a case-control study of type II diabetes (for study details, see [159]) and identify suggestive associations of putatively epistatic interactions.

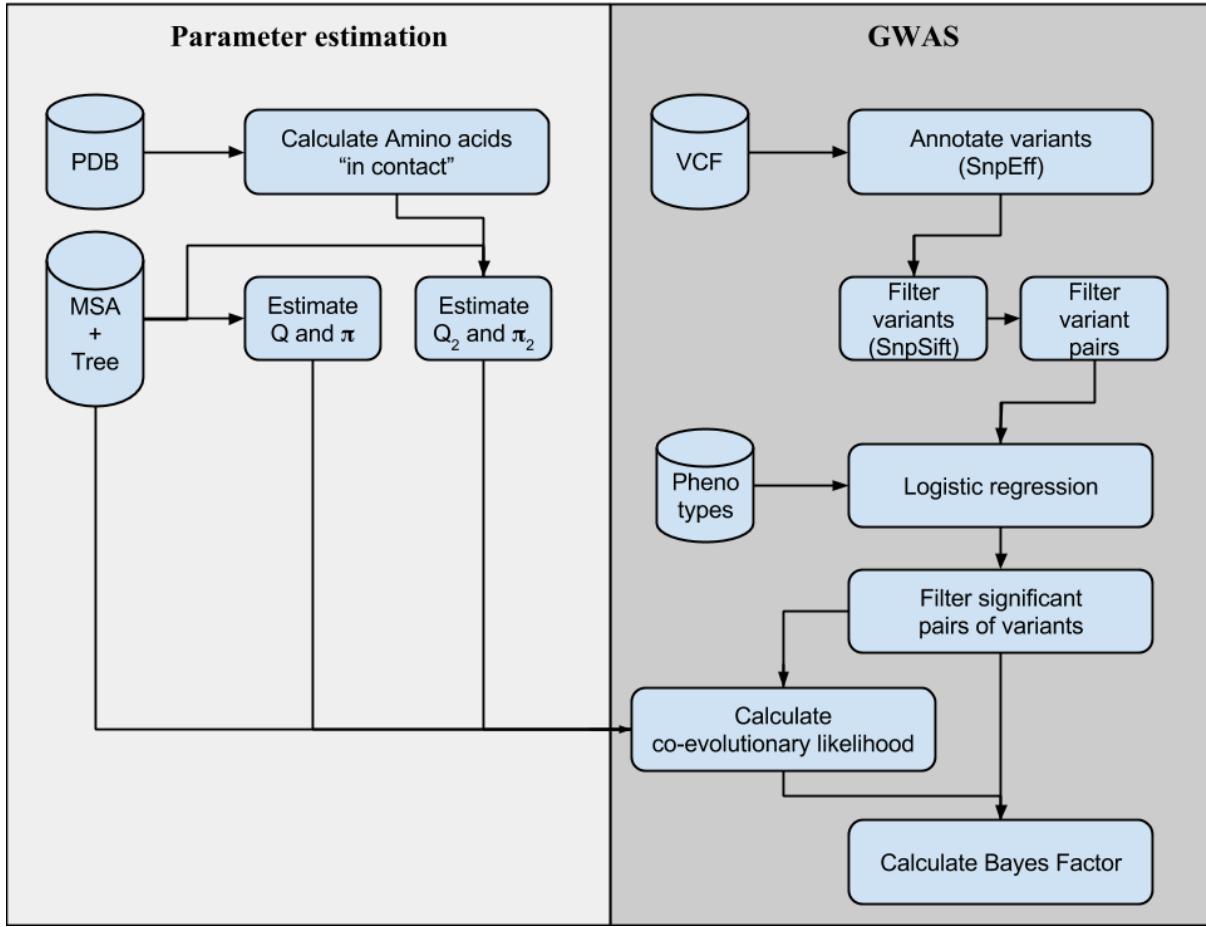


Figure 4.1: Analysis pipeline schematic

## 4.4 Methods

Our epistatic GWAS analysis pipeline involves three key steps, as shown in Figure 4.1. First, we learn a co-evolutionary substitution rate matrix from pairs of amino acids that are known to be in contact within proteins. Second, we analyse a GWAS data set to identify pairs of non-synonymous variants that show (possibly weak) evidence of epistasis. Third, for each pair of variants identified in step 2, we measure the evidence of co-evolution of the pair of encoded amino acids, and combine it with the GWAS evidence by calculating a joint Bayes factor.

#### 4.4.1 Substitution model for pairs of interacting amino acids

In this section, we describe how we estimate two substitution rate matrices: i) the first is the usual  $20 \times 20$  substitution rate matrix  $Q$  describing the evolution of individual amino acids; ii) the second,  $Q_2$ , is a  $400 \times 400$  substitution rate matrix for pairs of interacting residues.

We use the 100-way vertebrate multiple sequence alignment and accompanying phylogenetic tree  $T$  available from the UCSC Genome Browser [123]. This alignment includes the DNA sequences of 100 species whose genome is completely or nearly completely sequenced, with 12 primates, 44 non-primate eutherians, 5 marsupials, 14 birds, 6 reptiles, 16 ray-finned fish and 8 lobe-finned fish. The multiple alignment is performed using the multiz algorithm [22, 126].

From the  $\sim 21,000$  human protein structures (resolution less than  $3 \text{ \AA}$ ) available in Protein Data Bank, we extracted a set of  $\sim 770,000$  pairs of “within protein interaction” residues, defined as pairs of amino acids from the same protein where at least one pair of atoms is within  $3 \text{ \AA}$ . Similarly, from the set of  $\sim 5,700$  models of co-crystallized complexes in PDB, we extracted a set of  $\sim 12,000$  pairs of “protein-protein interacting” residues, defined as amino acids from different proteins that satisfy the same distance criterion.

To derive the rate matrix  $Q$ , we consider the complete set of  $n \sim 22 \times 10^6$  protein coding sites present in the alignment, irrespective of the presence or absence of contacts.  $Q$  is obtained following classical sequence evolution theory ([254, 80]). First, for each pair of species  $s_i$  and  $s_j$ , we obtain  $c_i(a)$  defined as the count of amino acid  $a$  in species  $s_i$ , and  $c_{i,j}(a,b)$  defined as the number of sites that have had a transition from amino acid  $a$  in  $s_i$  to  $b$  in  $s_j$ . The stationary probability of amino acid  $a$  in genome  $s_i$  is then defined as  $\pi_i = c_i(a)/n$ . Assuming a time reversible model, we get the frequency of change from  $a$  to  $b$ :  $f_{i,j}(a,b) = f_{j,i}(a,b) = (c_{i,j}(a,b) + c_{j,i}(a,b))/(2n)$ . Let  $P_{i,j}$  be the amino acid transition probability matrix from  $s_i$  to  $s_j$ , i.e.  $P_{i,j}(a,b)$  is the probability that species  $s_j$  has amino acid  $b$  given that species  $s_i$  has amino acid  $a$ . Then  $P_{i,j}$  is obtained through the relation  $f_{i,j}(a,b) = \pi_i(a) \cdot P_{i,j}(a,b)$ , or  $P_{i,j}(a,b) = f_{i,j}(a,b)/\pi_i(a)$ . Let  $t_{i,j}$  be the total branch length

between  $s_i$  and  $s_j$  (obtained from the phylogenetic tree). Assuming time reversibility, we have  $P_{i,j} = e^{Q \cdot t_{i,j}}$ , and thus  $Q = \log[P_{i,j}/t_{i,j}]$  [254]. Taking into account the estimation error, the equation becomes  $\hat{Q}_{i,j} = \log[P_{i,j}/t_{i,j}] + \epsilon_{i,j}$ , where  $\epsilon_{i,j}$  is an error matrix. Under the assumption that the mean error is zero, we can approximate the rate matrix by the calculating an average of all estimates:

$$\begin{aligned}\hat{Q} &= \frac{1}{N(N-1)/2} \sum_{i < j} \hat{Q}_{i,j} \\ &= \frac{2}{N(N-1)} \sum_{i < j} \frac{1}{t_{i,j}} \log[\hat{P}_{i,j}]\end{aligned}$$

The much larger substitution matrix  $Q_2$  describes the substitution rate from any pair of amino acids  $(a, b)$  to any other pair  $(c, d)$ . It is derived similarly to  $Q$ , but considering only pairs of amino acids from the set of within protein interacting pairs of amino acids. We only take into account amino acids pairs within the same chain, that are separated by 20 amino acids or more.

#### 4.4.2 Calculating likelihood of individual and pairs of alignment columns

Given a substitution rate matrix  $Q$ , a multiple sequence alignment MSA, and a phylogenetic tree  $T$  for the sequences in MSA, the likelihood of an alignment column  $MSA(i)$  (i.e.  $L_1[MSA(i)]$ ) can be calculated using Felsenstein's algorithm [80], which has a time complexity of  $O(N \cdot |\Sigma|^2)$ , where  $|\Sigma| = 20$  is the alphabet size and  $N$  is the number of sequences in the alignment. Given matrix  $Q_2$ , the same algorithm can be used to compute the likelihood  $L_2[MSA(i), MSA(j)]$  of a pair of alignment columns  $(MSA(i), MSA(j))$ , but now in time  $O(N \cdot |\Sigma|^4)$ .

A test for co-evolution of two positions  $i, j$  from the same or different proteins is obtained

from the log-likelihood ratio of these two models:

$$\ell_C[MSA(i), MSA(j)] = \log \left[ \frac{L_2[MSA(i), MSA(j)]}{L_1[MSA(i)] \cdot L_1[MSA(j)]} \right] \quad (4.1)$$

where the denominator assumes that the amino acids  $i$  and  $j$  evolve independently.

In a GWAS study these likelihood calculations need to be performed on a very large number of pairs of sites, thus optimizations are required to ensure manageable running time. First, pre-calculation of matrix exponentials  $P(t) = e^{Qt}$  can be performed for all values of  $t$  corresponding to individual branch lengths in the phylogenetic tree  $T$ . Second, “constant-tree caching” is used to cache likelihood values for sub-trees of the phylogenetic tree where all nodes have the same amino acid.

#### 4.4.3 GWAS model

Consider a GWAS with  $N_S$  samples (individuals) and  $N_V$  variants, we use the standard notation for phenotypes and code them as  $d_s = 1$  when individual  $s$  is affected by disease (cases) and  $d_s = 0$  if the individual is “healthy” (control). Let  $\bar{d} = [d_1, \dots, d_{N_S}]$  be a phenotype vector and  $g_{s,i} \in \{0, 1, 2\}$  a genomic variant for sample  $s$  at locus  $i$ . A logistic model of disease risk [13] is

$$\begin{aligned} p_{s,i} &= P(d_s = 1 | g_{s,i}, \bar{\theta}) \\ &= \phi(\theta_0 + \theta_1 g_{s,i} + \theta_2 c_{s,1} + \theta_3 c_{s,2} + \dots) \\ &= \frac{1}{1 + e^{-(\theta_0 + \theta_1 g_{s,i} + \theta_2 c_{s,1} + \theta_3 c_{s,2} + \dots)}} \\ &= \phi(\bar{\theta}^T \bar{g}_{s,i}) \end{aligned}$$

where  $\phi(\cdot)$  is the sigmoid function,  $c_{s,1}, c_{s,2}, \dots$  are covariates for each individual  $s$  (these covariates usually include sex, age and eigenvalues from population structure analysis [185]),  $\bar{g}_{s,i} = [1, g_{s,i}, c_{s,1}, c_{s,2}, \dots, c_{s,N_C}]$ , and  $\bar{\theta} = [\theta_1, \theta_2, \dots, \theta_m]$ . The parameter estimates  $\bar{\theta}$  are ob-

tained by solving the maximum likelihood equation

$$\begin{aligned}
L(\bar{\theta}) &= \prod_{s=1}^{N_S} P(d_s | \bar{\theta}, g_{s,i}) \\
&= \prod_{s=1}^{N_S} p_{s,i}^{d_s} (1 - p_{s,i})^{1-d_s} \\
&= \prod_{s=1}^{N_S} \phi(\bar{\theta}^T \bar{g}_{s,i})^{d_s} (1 - \phi(\bar{\theta}^T \bar{g}_{s,i}))^{1-d_s}
\end{aligned}$$

Using this model, we have two hypotheses: i) the null hypothesis,  $H_0$ , assumes that genotype does not influence disease probability (i.e.  $\theta_1 = 0$ ); and ii) the alternate hypothesis,  $H_1$ , assumes that the genotype does influence disease probability (i.e.  $\theta_1 \neq 0$ ). We can compare these two hypotheses using a log likelihood ratio test, so we define

$$\ell_G = \log \left[ \frac{L(\bar{\theta}|H_1)}{L(\bar{\theta}'|H_0)} \right]$$

where  $\bar{\theta}'$  and  $\bar{\theta}$  are the maximum likelihood estimates for null and alternate models respectively. According to Wilk's theorem [247], the log likelihood ratio has a  $\chi_1^2$  distribution under the null hypothesis, so we can easily calculate a p-value.

Next, we extend the logistic model to accommodate interacting loci. For an individual (sample  $s$ ), we model interactions between two genetic loci  $i$  and  $j$ , having genotypes  $g_{s,i}$  and  $g_{s,j}$ , by extending the logistic model

$$P(d_s | g_{s,i}, g_{s,j}, H_1) = \phi[\theta_0 + \theta_1 g_{s,i} + \theta_2 g_{s,j} + \theta_3(g_{s,i}g_{s,j})] \quad (4.2)$$

$$+ \theta_4 c_{s,1} + \dots + \theta_m c_{s,N_{cov}} \quad (4.3)$$

$$= \phi(\bar{\theta}^T \bar{g}_{s,i,j}) \quad (4.4)$$

where  $\bar{g}_{s,i,j} = [1, g_{s,i}, g_{s,j}, (g_{s,i}g_{s,j}), c_{s,1}, c_{s,2}, \dots, c_{s,N_{cov}}]^T$ . An implicit assumption in this equation is that  $g_{s,i}$  and  $g_{s,j}$  are not correlated (e.g. they are not located in the same LD-Block),

which hinders the application of this model for detecting interactions within proteins. This can be enforced either by using haplotype structure information (e.g. from HapMap) or by limiting the application of the model to variants either in different chromosomes or sufficiently distant (say  $> 1MB$ ), we use the latter. The null hypothesis  $H_0$  assumes that variants act independently

$$P(d_s|g_{s,i}, g_{s,j}, H_0) = \phi[\theta'_0 + \theta'_1 g_{s,i} + \theta'_2 g_{s,j} + \theta'_3 c_{s,1} + \dots] \quad (4.5)$$

$$= \phi(\bar{\theta}'^T \bar{g}'_{s,i,j}) \quad (4.6)$$

where  $\bar{g}'_{s,i,j} = [1, g_{s,i}, g_{s,j}, c_{s,1}, c_{s,2}, \dots, c_{s,N_{cov}}]^T$ .

Just as in the single loci, this requires fitting the logistic regression parameters, thus we investigated several algorithms for logistic regression parameter fitting. The fastest convergence is obtained using Iterative Reweighted Least Squares (IRWLS [64]) and Broyden-Fletcher-Goldfarb-Shanno algorithm (BFGS [28]) with some code optimizations. In most cases, IRWLS converges faster, so it was selected as the default implementation in our analysis.

Another way to compare the null hypothesis to the alternative hypothesis, is using a Bayesian formulation [124, 237]

$$\begin{aligned} P(H_1|\mathcal{D}) &= \frac{P(\mathcal{D}|H_1)P(H_1)}{P(\mathcal{D})} = \frac{\int P(\mathcal{D}|\bar{\theta}, H_1)P(\bar{\theta}|H_1)P(H_1)d\bar{\theta}}{P(\mathcal{D})} \\ \Rightarrow \frac{P(H_1|D)}{P(H_0|D)} &= \frac{\int P(\mathcal{D}|\bar{\theta}, H_1)P(\bar{\theta}|H_1)d\bar{\theta}}{\int P(\mathcal{D}|\bar{\theta}', H_0)P(\bar{\theta}'|H_0)d\bar{\theta}'} \frac{P(H_1)}{P(H_0)} = BF \frac{P(H_1)}{P(H_0)} \end{aligned}$$

where  $BF$ , the ratio of the two integrals, is the Bayes factor. Using a Bayesian formulation has two main advantages: i) the hypothesis are automatically corrected for model complexity since Bayes factor asymptotically converges to Bayesian Information Criteria (BIC) [124],

and ii) we can compare non-nested models. The Bayes factor for the epistatic model becomes:

$$BF_G = \frac{\int \prod_{s=1}^{N_S} \phi(\bar{\theta}^T \bar{g}_{s,i,j})^{d_s} [1 - \phi(\bar{\theta}^T \bar{g}_{s,i,j})]^{1-d_s} P(\bar{\theta}|H_1) d\bar{\theta}}{\int \prod_{s=1}^{N_S} \phi(\bar{\theta}'^T \bar{g}'_{s,i,j})^{d_s} [1 - \phi(\bar{\theta}'^T \bar{g}'_{s,i,j})]^{1-d_s} P(\bar{\theta}'|H_0) d\bar{\theta}'} \quad (4.7)$$

Calculating Bayes factors is challenging because most of the times there are no closed form equations and even if the integrals can be computed by means of numerical algorithms, it imposes a significant computational burden thus making it impractical for large datasets such as GWAS data. We can approximate the integrals using Laplace's method [124]. If  $g(x)$  has a maximum at  $x_0$ , it can be shown that

$$\int e^{-\lambda g(x)} h(x) dx \simeq h(x_0) e^{\lambda g(x_0)} \sqrt{\frac{2\pi}{\lambda g''(x_0)}}$$

The multivariate case for  $\bar{x} \in \Re^d$  is an analogous extension that uses a Hessian matrix instead of a second derivative of  $g(\cdot)$

$$\int e^{\lambda g(\bar{x})} h(\bar{x}) d\bar{x} \simeq h(\bar{x}_0) e^{\lambda g(\bar{x}_0)} \left( \frac{2\pi}{\lambda} \right)^{d/2} \left[ \frac{\partial^2 g(\bar{x})}{\partial \bar{x} \partial \bar{x}^T} \right]^{-1/2} \quad (4.8)$$

Using equation 4.8 we can try to approximate the improper integrals in equation 4.7 by the transformation  $L(\bar{\theta}) = e^{\ell(\bar{\theta})}$ , where  $\ell(\cdot)$  is the log-likelihood of the data, so we can use Laplace approximation by applying Eq.4.8 at the point of the maximum likelihood. In order to do so, we need to calculate the Hessian matrix in Eq.4.8. Fortunately in logistic models , it can be shown that for genotype terms

$$\frac{\partial^2 \ell(\bar{\theta})}{\partial \theta_i \partial \theta_j} = \sum_s g_{s,i} g_{s,j} p_s (1 - p_s)$$

Using analogous derivation for the covariates, we can find an analytic form of the Hessian, which completes the Laplace approximation formula.

Calculating Bayes factors involves using prior parameter distributions. In order to estimate these distributions, we run the logistic regression fitting analysis and plot the parameter distributions for different levels of significance. As expected most parameters have uni-modal distribution, except for  $\theta_3$ , which has a multi-modal distribution. For all parameters, except  $\theta_3$ , we use a normal distribution centred at the mean and variance set to one ( $\sigma = 1$ ) even though most times the variance is much smaller. This is done to avoid penalizing outliers too heavily and to have smooth derivatives near the maximum likelihood estimates. For  $\theta_3$ , we use a Gaussian bimodal distribution fitting the parameters by means of an EM algorithm.

**Computational and statistical issues** The computational burden for the detection of pairs of interacting genetic loci affecting disease risk is significantly larger than in a standard (single variant) GWAS study. A priori all pairs of variants should be analysed, thus significantly increasing the number of statistical tests. This also reduces the statistical power since the required p-value significance level would be orders of magnitude smaller. A naïve approach would estimate that if a typical genetic sequencing study has  $10^6$  variants, a GWAS on epistatic variant pairs would square that number of statistical tests, thus p-values required for significance would be in the order of  $0.05/(10^6)^2 = 5 \cdot 10^{-14}$ .

Fortunately the number of tests can be reduced significantly. First, in this study, we only concentrate on non-synonymous coding variants. Second, as required by our co-evolutionary model, only variants overlapping a multiple sequence alignment are taken into account (when several multiple sequence alignments overlapped a region, the alignment with the longest number of proteins was selected). Third, if two variants  $g_i$  and  $g_j$  are such that the interaction term ( $g_{s,i}g_{s,j}$ ) is zero in all samples, which usually happens for pairs of rare variants, then  $BF_G = 1$  (according to our estimates, this filters out over 95% of variant pairs). Fourth, if the variants and the epistatic term  $[g_{s,i}, g_{s,j}, g_{s,i}g_{s,j}]$  are linearly dependent, the logistic regression equations are undetermined, so we skip such variant pairs. Fifth, if one of the variants has high allele frequency with respect to the other, all non-zero epistatic terms

may lie in the same positions as non-zero genotypes from the low frequency variant, causing logistic regression estimates to artificially inflate the coefficients of the low frequency variant and the epistatic term thus creating an artificially high association (low p-value). So we filter out these variant pairs as well. Finally, we filter out variants having Hardy-Weinberg p-value  $10^{-6}$  or less since a low p-value might indicate a sequencing quality problem. Once the results are obtained, we can focus on pure epistatic interactions (i.e. interactions with no marginal contribution from each independent loci) by further filtering results and keeping variant pairs whose alternative logistic model (see equation 4.2) has small absolute values for  $\theta_1$  and  $\theta_2$  while having large absolute values for  $\theta_3$ , specifically we keep results if  $|\theta_3| > K(|\theta_1| + |\theta_2|)$  (based on empirical data, we set  $K = 3$ ).

#### 4.4.4 Putting it all together

In summary, we first calculate the transitions matrices for the Markov models ( $Q$  and  $Q_2$ ) based on observations from protein structures (PDB) and multiple sequence alignments (UCSC's 100-way). We analyse variants from genome sequencing data first by filtering only for non-synonymous variants, then analysing all possible pairs of variants and filtering out those that are unsuitable for further analysis. From the pairs of variants that pass filtering, we fit two logistic regression models (null and alternative hypothesis), then calculate a p-value using the log-likelihood ratio, and keeping pairs of variants having p-values below a predefined threshold ( $10^{-6}$ ). These pairs of variants are then analysed under our co-evolutionary model, we find the corresponding columns in the multiple sequence alignment and calculate the likelihoods for the null and alternative models by means of Felsenstein's algorithm (using matrices  $Q$  and  $Q_2$  respectively). Finally, likelihoods from co-evolutionary and logistic regression models are used to calculate the total Bayes Factor ( $BF_T$ ) by means

of Laplace's approximation:

$$BF_T = \frac{\int \prod_{s=1}^{N_S} \phi(\bar{\theta}^T \bar{g}_{s,i,j})^{d_s} [1 - \phi(\bar{\theta}^T \bar{g}_{s,i,j})]^{1-d_s} P(\bar{\theta}|H_1) d\bar{\theta}}{\int \prod_{s=1}^{N_S} \phi(\bar{\theta}'^T \bar{g}'_{s,i,j})^{d_s} [1 - \phi(\bar{\theta}'^T \bar{g}'_{s,i,j})]^{1-d_s} P(\bar{\theta}'|H_0) d\bar{\theta}'} \cdot \frac{L_2[MSA(i), MSA(j)]}{L_1[MSA(i)] \cdot L_1[MSA(j)]}$$

$$BF_T = BF_G \cdot L_C$$

## 4.5 Results

Our approach, which is summarized in Figure 4.1, involves three main components. First we estimate evolutionary substitution rates for individual amino acids in a protein as well as for pairs of amino acids (either from the same protein or not) that are physically interacting. Given a set of multiple protein sequence alignment these evolutionary models can be used as a statistical test for co-evolution between any two amino acids without using structural information. Second, a statistical test for epistasis is developed to identify pairs of non-synonymous variants that show (often weak) evidence of interaction in the way they associate to a given trait. Finally, evidence from the co-evolution model and the epistatic GWAS model are combined in a Bayesian framework.

### 4.5.1 Co-evolutionary substitution models

The approach described in Methods was used to obtain substitution rate matrices  $Q$  for individual amino acids and  $Q_2$  for pairs of physically interacting residues within the same protein. Unsurprisingly,  $Q$  (or more precisely a transition matrix  $P(t)$  obtained from  $Q$ ) is very similar to well known transitions matrices such as PAM [66].

The structure of  $Q_2$ , which describes substitution rates between one pair of interacting amino acids to another, is richer. Of particular interest are pairs of amino acids having high ratio  $R(ab, cd) = Q_2(ab, cd)/(Q(a, c) \cdot Q(b, d))$  which is an indicative of co-evolution. For example the highest ratio is found in amino acid pair transition V.I  $\rightarrow$  W.W (i.e. amino acid

V mutated to W in one of the sequences, and amino acid I changed to W in the other). In fact all top 10 ratios are transitions to W.W amino acid pairs. This makes sense considering that tryptophan pairs are well known  $\beta$ -hairpin stabilizers and are considered as a paradigm for designing stable  $\beta$ -hairpins [205]. Nevertheless, we should keep in mind that tryptophan is: i) the least frequent amino acid in the genome (having frequency of 1.1% and being encoded in only by TGG codon), and ii) is usually in the core of the protein, as opposed to being part of surface interaction sites. These facts may indicate that the high  $R(ab, cd)$  ratio may be caused by a bias when estimating the model based on within-protein interactions. Unfortunately, at the moment there is no enough data from co-crystallized proteins in PDB to estimate the parameters from true interacting loci.

Another type of pair transitions with large  $R(ab, cd)$  ratio is the double transitions to a pair of phenylalanine amino acids from a pair of hydrophobic amino acids (Lysine, Asparagine, Glutamine, Arginine, Aspartic acid and Glutamic acid). Phenylalanine-Phenylalanine interaction pairs are assumed to conform  $\pi - \pi$  interactions which are predicted and experimentally observed to be energetically favourable [112].

#### 4.5.2 Co-evolutionary model validation

We first assessed the ability of our co-evolutionary model to detect interacting sites located within the same protein by computing the likelihood ratio of candidate pairs of sites under a co-evolutionary model ( $Q_2$ ) versus under independence ( $Q$ ). Although such pairs of sites are unlikely to exhibit evidence of epistasis in GWAS studies (due to linkage), accurate prediction of interacting sites in a given protein are useful for many other purposes, such as protein structure prediction and prediction of the impact of individual mutations. Figure 4.2 shows that interacting sites tend to have higher likelihood ratio scores than non-interacting ones (Mann-Whitney p-value  $< 2.2 \times 10^{-16}$ ). Although the likelihood ratio score by itself cannot perfectly discriminate between the two classes, only 25.9% of non-interacting pairs have a likelihood ratio above the median likelihood ratio of interacting pairs. Figure 4.3

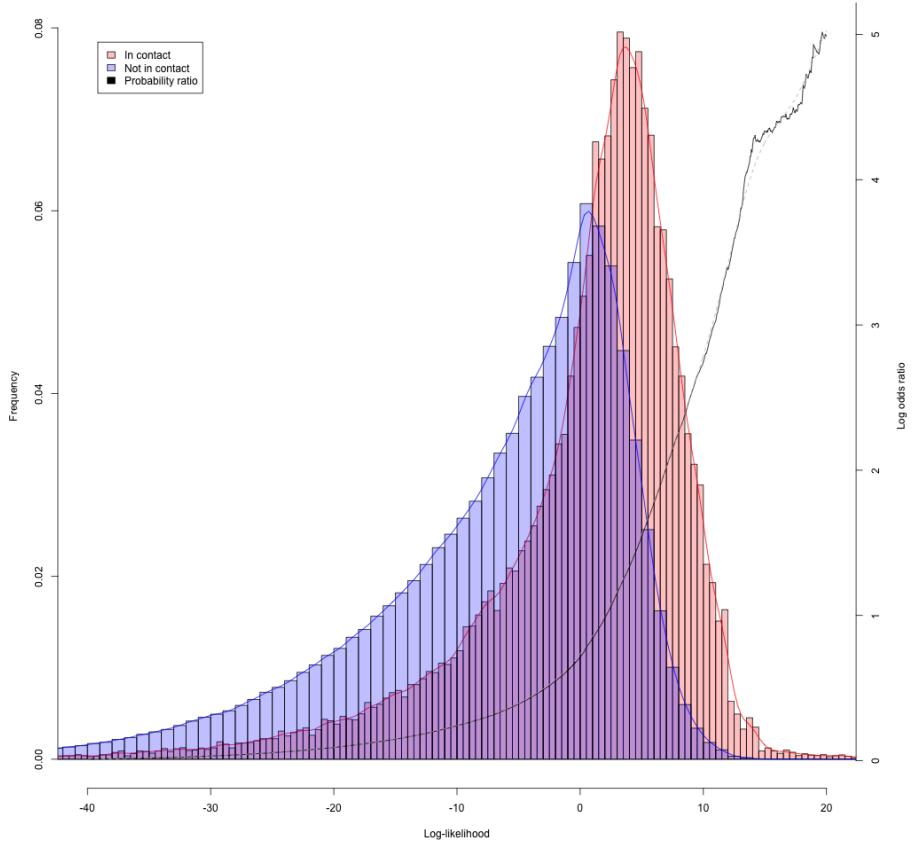


Figure 4.2: Histogram of log-likelihood values of pairs of amino acids in contact (red) and not in contact (blue) for amino acids within the protein (PDB). Log-odds of contacting vs non-contacting pairs (black) and smoothed log-odds (dotted grey).

shows a receiver operator curve (ROC) curve for a classifier based on our method.

To confirm that a co-evolutionary model estimated on pairs of interacting sites from the same protein is useful at predicting pairs of interacting sites between proteins, we repeated the same type of analysis for  $\sim 3,000$  pairs of interacting ( $< 3 \text{ \AA}$ ) and  $\sim 3,000$  pairs of non-interacting ( $> 30 \text{ \AA}$ ) residues from distinct proteins, obtained from co-crystal structures in PDB (see Methods). As seen on Figure 4.4, the two classes of sites have substantially different likelihood ratio distributions (Mann-Whitney one sided test:  $p - value < 2.2 \times 10^{-16}$ ), although slightly less so than for sites from the same protein. Only 29% of non-interacting sites have a likelihood ratio larger than the median for interacting sites. These empirical

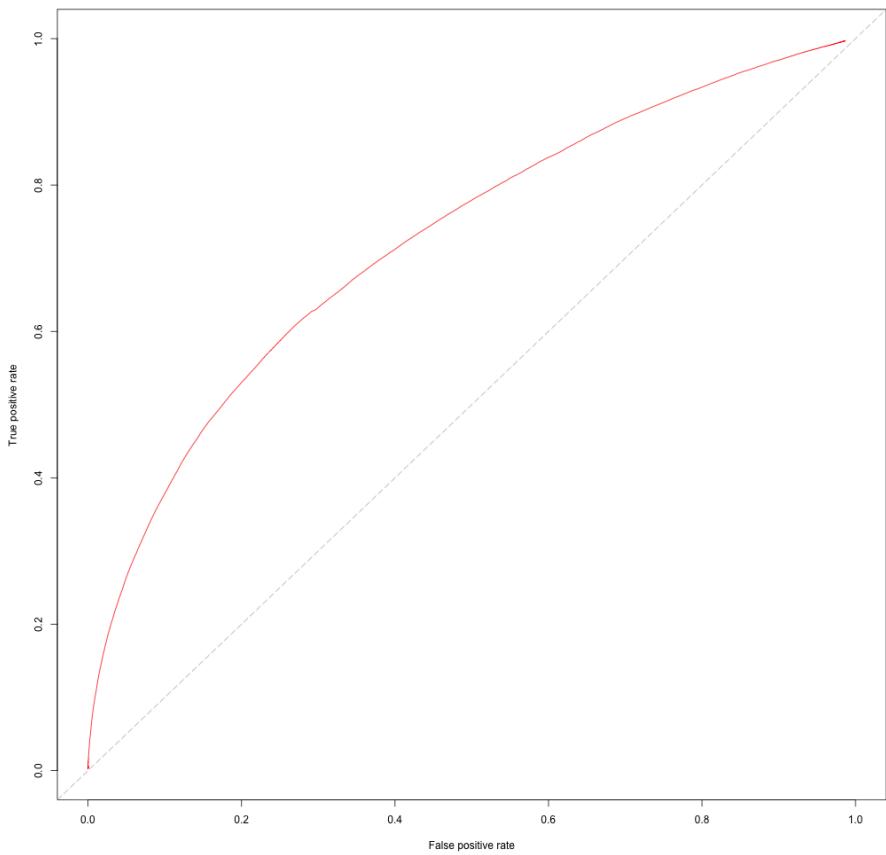


Figure 4.3: Classifier's receiver operator curve (ROC).

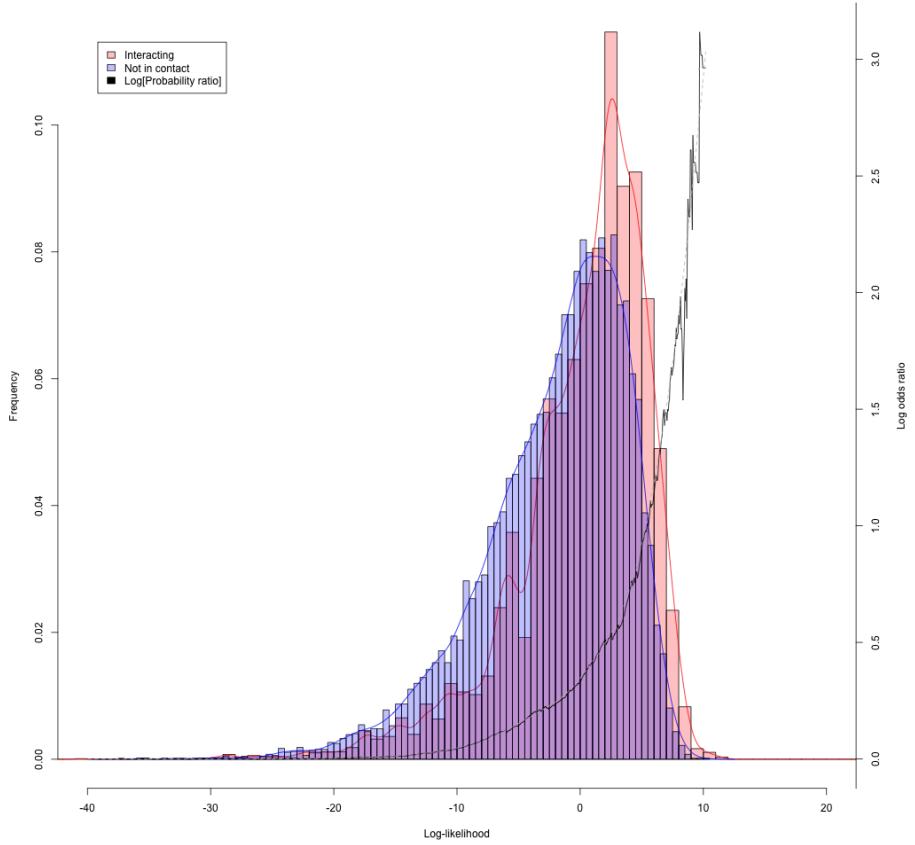


Figure 4.4: Histogram of log-likelihood values of pairs of amino acids in contact (red) and not in contact (blue) for amino acids in different proteins (co-crystallized entries from PDB). Log-odds of contacting vs non-contacting pairs (black) and smoothed log-odds (dotted grey)

distributions allow us to approximate of the log odds of the “interacting” vs “non-interacting” amino acids distributions as

$$\begin{aligned}\ell_{odds}(x) &= \log \left[ \frac{P[L_2(MSA(i), MSA(j)) \geq x]}{P[L_1(MSA(i)) \times L_1(MSA(j)) \geq x]} \right] \\ &\simeq e^{\alpha x} - \beta\end{aligned}$$

where  $\alpha = 0.195$  and  $\beta = 1.018$  (in order to avoid biases, the log odds value is capped to 4.0).

Figure 4.5 shows the example of a predicted contact  $\ell_C = 7.7$  between *Senp1* and *Sumo1* proteins detected by our method. The co-crystallized structure from PDB highlights the interacting amino acids (less than 3 Å apart) and the corresponding multiple alignment columns.

Although our approach aims at identifying contacting residues from different proteins, it can also be used to predict the presence or absence of interactions between proteins as a whole. We extracted from BioGrid [220] a set of  $\sim 3,000$  pairs of human proteins with evidence of interaction, and further required that both proteins belong to the same pathway (MsigDb, C2 groups [223]), and their corresponding genes are expressed in the same tissue (GTex [151], expression of 1 FPKM or more, tissues  $\in \{\text{skeletal muscle, adipose tissue, pancreatic Islets, liver}\}$ ). We randomly selected as “non-interacting” pairs the same number of pairs amongst those that do not fulfil any of the three conditions.

Let the two proteins considered have amino acid sequences  $A = a_1 \dots a_m$  and  $B = b_1 \dots b_n$ . To obtain the prediction score for this pair of proteins, we identify the pair of length- $k$  substrings  $a_i, a_{i+1}, \dots, a_{i+k-1}$  and  $b_j, b_{j+1}, \dots, b_{j+k-1}$  that exhibit the strongest support for parallel or anti-parallel interactions

$$\ell_C(k) = \max \left[ \sum_{l=0}^{k-1} \ell_C[MSA(a_{i+l}), MSA(b_{j+l})], \sum_{l=0}^{k-1} \ell_C[MSA(a_{i+l}), MSA(b_{j+k-1-l})] \right]$$

where  $k = 3$  was determined empirically to provide the best predictive power. As shown in Figure 4.8), prediction accuracy is quite good ( $p\text{-value} < 2 \cdot 10^{-42}$ ), taking into account the modest amount of information considered by the model.

### 4.5.3 Epistatic GWAS analysis

We applied our methods to a cohort of  $\sim 13,000$  individuals in a case-control study of type II diabetes [159]. This multi-ethnic study covers exons of unrelated individuals from five major ancestral groups (European descent, South Asian, East Asian, Hispanic and African

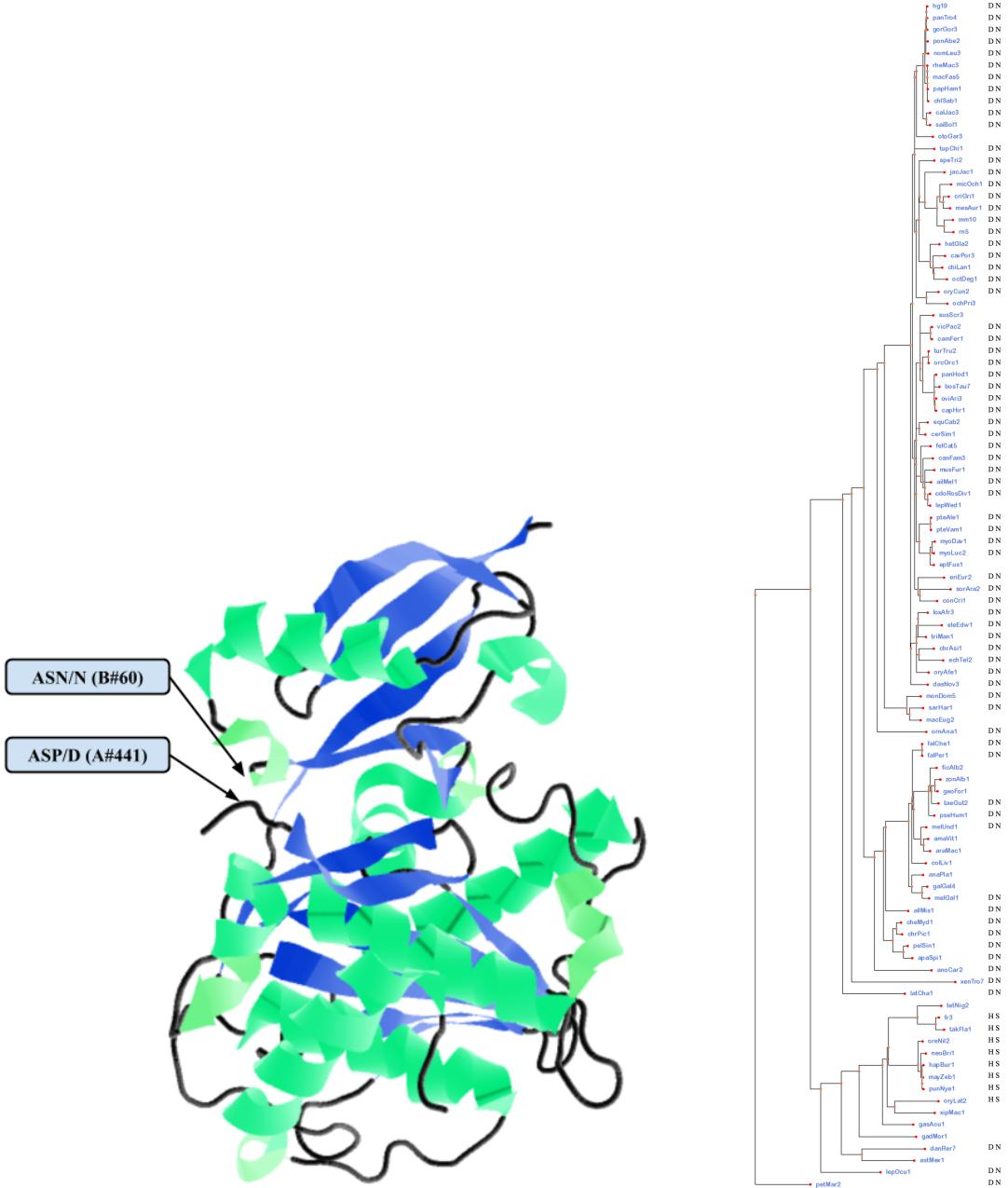


Figure 4.5: Example of interaction between amino acid #441 of *Senp1* and #60 of *Sumo1* proteins detected by our method with  $\ell_C = 7.7$ . Left: PDB structure 2G4D, shows that the amino acids are in close proximity. Right: Multiple sequence alignment and phylogenetic tree showing the putative compensatory amino acid substitution pair D.N replaced by H.S.

American descent) using an average sequencing coverage over  $80\times$ , yielding 1.7 million coding variants. The filters described in Methods section resulted in a number of variant pairs being analysed of less than 50 million. By means of the z-score relationship between Bayes Factor and p-values shown in [95], we can set the GWAS significance threshold for 50 million pairs at  $\log_{10}[BF_T] = 9.0$ .

**Results** Variants annotated and filtered according to the previous paragraphs resulted in the identification of 689,186 variant pairs having log likelihood  $\ell_G$  above 6 (see equation 4.2) that were further analysed under co-evolutionary and Bayesian models yielding 303 pairs with log Bayes Factor  $\log_{10}[BF_T]$  over 9.0. The complete analysis took less than 2 days using a 1,000 CPU-cluster, thus showing that an epistatic GWAS analysis is feasible using current computational resources. Table 4.6 shows the main results from our GWAS epistatic analysis. Genes highlighted in red are associated with diabetes or known to be in diabetes-related pathway. It should be noted that some of the top results include amino acid modification sites such as Phosphoserine (or Glycosylation, not shown), which are likely to be interaction loci.

**Associations are purely epistatic** In order to understand the nature of the putative epistatic associations, we compared our top epistatic GWAS results against a large single marker GWAS study of type II diabetes from the DIAGRAM consortia [256, 235] as well as a meta analysis from the same consortia [165]. For each of our top 1,000 results we looked up the closest entry from the DIAGRAM dataset (i.e. the entry having smallest distance, as measured in number of bases) and analysed the corresponding p-values from the three publicly released DIAGRAM datasets.

Only 14 of the top 1,000 results show suggestive association ( $p\text{-value} \leq 10^{-4}$ ) in a nearby loci (average distance 977 bases). Of these, only one of them is in our top 10 results (8:30982425\_T/G variant in *WRN* gene) has DIAGRAM p-value of  $4.6 \times 10^{-4}$  and odds ratio 1.06. Neither of the other 13 suggestive single marker variants are our top 50 results.

Variant 1			Variant 2			Logistic regression log10(BF)	p-value	Co-evolutionary Log-Likelihood	Combined model log10(BF)
Coordinate	Gene	Functional annotation	Coordinate	Gene	Functional annotation				
4:90743415_T/C	SNCA	NON_SYNONYMOUS_CODING	2:179659911_G/A	TTN	NON_SYNONYMOUS_CODING	6.47	2.73E-06	1.50	7.12
3:5321690_G/C	PRKCD	NON_SYNONYMOUS_CODING	14:75746689_C/T	FOS	NON_SYNONYMOUS_CODING	6.43	2.26E-06	2.11	7.35
2:242795125_G/A	PDCD1	NON_SYNONYMOUS_CODING	8:13356801_G/A	DLC1	NON_SYNONYMOUS_CODING	6.36	2.62E-06	1.01	6.80
11:57582923_G/A	CTNND1	AA_modification:Phosphoserine	2:220420784_G/A	OBSL1	NON_SYNONYMOUS_CODING	6.22	3.85E-06	0.29	6.35
1:12298763_T/C	DDX20	NON_SYNONYMOUS_CODING	3:125826058_T/C	ALDH1L1	NON_SYNONYMOUS_CODING	6.07	6.60E-06	4.62	8.08
2:179457146_G/A	TTN	NON_SYNONYMOUS_CODING	22:35695930_C/A	TOM1	NON_SYNONYMOUS_CODING	6.04	4.77E-06	2.72	7.22
1:45797504_C/G	MUTYH	NON_SYNONYMOUS_CODING	8:30982425_T/G	WRN	NON_SYNONYMOUS_CODING	6.00	9.62E-06	2.74	7.20
16:4855278_A/G	GLYR1	AA_modification:Phosphoserine	8:13259100_G/A	DLC1	NON_SYNONYMOUS_CODING	5.90	1.58E-05	3.57	7.45
11:45975129_C/T	PHF21A	NON_SYNONYMOUS_CODING	19:49456190_C/A	BAX	NON_SYNONYMOUS_CODING	5.81	1.55E-05	2.31	6.81
7:128490102_G/A	FLNC	NON_SYNONYMOUS_CODING	8:13357339_G/C	DLC1	NON_SYNONYMOUS_CODING	5.81	1.02E-05	6.60	8.67
11:236090_G/A	SIRT3	NON_SYNONYMOUS_CODING	4:110615838_C/T	CASP6	NON_SYNONYMOUS_CODING	5.79	9.91E-06	1.44	6.42
8:144993930_C/G	PLEC	NON_SYNONYMOUS_CODING	14:73422258_C/G	DCAF4	NON_SYNONYMOUS_CODING	5.79	1.08E-05	4.79	7.87
1:45224997_A/C	KIF2C	NON_SYNONYMOUS_CODING	2:108921032_C/T	SULT1C2	NON_SYNONYMOUS_CODING	5.70	2.30E-05	4.98	7.86
11:134252895_C/T	B3GAT1	NON_SYNONYMOUS_CODING	15:75012984_T/C	CYP1A1	NON_SYNONYMOUS_CODING	5.68	1.56E-05	1.81	6.47
6:116441645_C/G	COL10A1	NON_SYNONYMOUS_CODING	20:30072135_G/A	REM1	NON_SYNONYMOUS_CODING	5.63	1.24E-05	0.08	5.67
1:201016295_G/A	CACNA1S	NON_SYNONYMOUS_CODING	19:17000695_G/A	F2RL3	NON_SYNONYMOUS_CODING	5.61	2.69E-05	4.12	7.40
7:43351409_T/G	HECW1	NON_SYNONYMOUS_CODING	2:219294200_A/G	VIL1	NON_SYNONYMOUS_CODING	5.59	8.39E-07	0.62	5.87
15:67457334_A/G	SMAD3	NON_SYNONYMOUS_CODING	1:201052381_A/G	CACNA1S	NON_SYNONYMOUS_CODING	5.58	2.34E-05	0.60	5.84
10:53822300_A/G	PRKG1	NON_SYNONYMOUS_CODING	9:140007465_G/A	DPP7	NON_SYNONYMOUS_CODING	5.56	1.46E-05	3.30	7.00
2:225362477_C/T	CUL3	NON_SYNONYMOUS_CODING	9:120476787_C/G	TLR4	STOP_GAINED	5.56	2.96E-05	3.16	6.93
2:220435835_G/C	OBSL1	NON_SYNONYMOUS_CODING	4:87735617_A/G	PTPN13	NON_SYNONYMOUS_CODING	5.55	1.26E-05	1.00	5.99

Table 4.6: Results from epistatic GWAS analysis of type II diabetes sequencing data. Genes form diabetes related pathways marked in red.

This indicates that our top results are populated by pure epistatic putative effects having little marginal association with disease risk. In light of this evidence, it should be noticed that *conditional search* methods (based on selecting the top single-marker results and performing epistatic analysis only on this subset) would have missed most of our top results. Likewise, an extension of that same limitation applies to our own method since we might be missing any pure epistatic interaction of order three above, a limitation that doesn't seem to be plausible to overcome without introducing prior biological knowledge of interacting loci (which is obviously not available at the moment).

#### 4.5.4 Power analysis

In order to assess our disease association power we performed extensive simulations. As it is often the case in this kind of analyses some simplifying but realistic assumptions were required to make simulations computationally tractable. We assumed that: i) the disease has a logistic risk model; ii) risk model has purely epistatic terms, i.e. that individual loci are assumed to have no marginal effect in disease risk; iii) disease prevalence is 8% according the well accepted prevalence for type II diabetes; iv) cofactors influencing disease such as population admixture, age and sex were perfectly reduced by the model, meaning that no residual effects remains after correction; v) genome wide significance was established as

$0.05/(10^6)^2 = 5.10^{-14}$ ; and vi) that the study has an equal number of cases and controls.

Under these assumptions we calculated power by running 100 iterations for every model having a combination of: i) sample sizes ranging from 10,000 to 1,000,000, ii) log odds disease risk ranging from 0.1 to 5, and iii) allele frequencies of each of the two loci ranging from 1% to 20%. Results for some selected models are shown in Figure 4.7.

As expected these simulations show that very large sample sizes are required to find epistatic effects on rare variant loci (i.e. allele frequencies below 1%). Even if the risk factor is assumed to be over 2, 3, or 5, sample sizes requirements are expected to be over 400,000; 200,000; and 100,000 respectively (see Figure 4.7, top-left plot). For common variants (i.e. allele frequency of 5%) the sample sizes look more attainable in the near future, ranging from 10,000 to 100,000 samples when risk factors decrease from 5 to 1.6 (Figure 4.7, middle row, left plot). Finally, for relatively common variants (allele frequency of 10%) the sample sizes up to 100,000 would be required for risk factors of 1.3, which is still considered a high risk factor (Figure 4.7, bottom right plot).

Unsurprisingly, our power calculation results show that large sample sizes are required in order to find epistatic interactions even under assumptions of relatively common variants and relatively high risk factors. This highlights how elusive finding epistatic genome wide associations can be.

## 4.6 Discussion

In this paper, we propose a novel methodology for genome wide association studies to identify pairs of variants under putative epistatic interaction. Due to the large number of statistical tests required in an epistatic GWAS analysis and the corresponding reduction of statistical power, this type of analysis is meant to be applied to datasets consisting of large number of samples. Our highly optimized algorithms are applicable to such large scale sequencing genomic studies and we show the application of our methods to a large scale exome sequencing

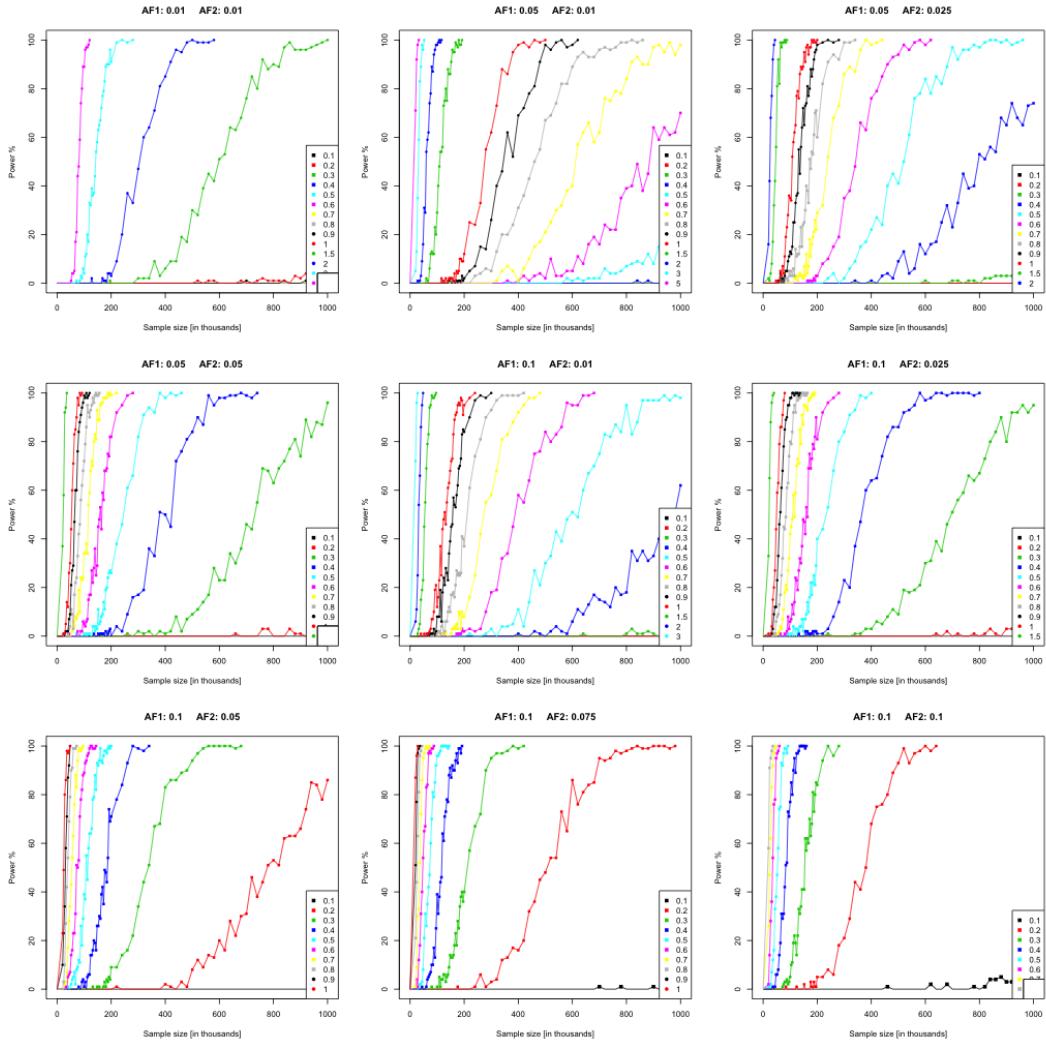


Figure 4.7: Power calculation using a case/control pure two-loci epistatic model for disease prevalences of 8% (type II diabetes). X-axis shows the number of samples in thousands (ranging from 10,000 to 1,000,000) and several joint risk values (from 0.1 to 5) are shown as different colors lines within each plot. Each plot represents a different allele frequencies combination (for each of the two loci), from top to bottom and left to right, the allele frequency value pairs are:  $[AF_1 = 1\%, AF_2 = 1\%]$ ,  $[AF_1 = 5\%, AF_2 = 1\%]$ ,  $[AF_1 = 5\%, AF_2 = 2.5\%]$ ,  $[AF_1 = 5\%, AF_2 = 5\%]$ ,  $[AF_1 = 10\%, AF_2 = 1\%]$ ,  $[AF_1 = 10\%, AF_2 = 2.5\%]$ ,  $[AF_1 = 10\%, AF_2 = 5\%]$ ,  $[AF_1 = 10\%, AF_2 = 7.5\%]$ ,  $[AF_1 = 10\%, AF_2 = 10\%]$

study for type II diabetes consisting of  $\sim 13,000$  samples and  $\sim 1,7M$  variants. First, this shows that it is indeed feasible to apply our methods to GWAS-scale datasets. Second, although larger cohorts are needed in order to find risk alleles that have lower frequencies and are not captured by this study, we show several suggestive association of pairs of putatively interacting variants affecting type II diabetes risk.

The co-evolutionary model we propose in section 4.4.1 requires multiple sequence alignment and the corresponding phylogenetic tree. Intuitively, using a MSA with larger number of sequences should improve co-evolutionary model detection and other co-evolutionary approaches indeed require very large MSA. Unfortunately, at this moment, MSA consisting of a large number of sequences are available only for a small fraction of all human proteins and they often consist of a mixture of ortholog and paralog sequences which may lead to biases in co-evolutionary models. Furthermore, both the phylogenetic tree and the number of sequences in the MSA should remain constant throughout the genome in order to take advantage of computational optimizations (matrix exponential pre-calculation and “constant tree caching”) that allow the algorithm to be applied at genome-wide scale. Many multiple sequence alignments (such as Pfam) have different number of sequences for each protein (thus different phylogenetic trees). This poses two main disadvantages for our methodology: i) we cannot benefit from the previously mentioned optimizations ; and ii) we would add the problem of reconciling different phylogenetic trees from two proteins, which may lead to inconsistencies. For all these reasons we selected UCSC’s multi-100way [123], a genome wide multiple sequence alignment of 100 vertebrates which has a single genome wide phylogenetic tree. This MSA is expected to grow with the advent of projects like G10K [104] thus enabling more precise co-evolutionary predictions.

In order to further validate our co-evolutionary model in the context of human disease, we tested whether  $\ell_C$  scores can separate clinically relevant variants from ClinVar database [134] according to their clinical significance attribute (CLNSIG). Interestingly, variants categorized as “benign” (i.e. non-pathogenic) or “druggable” (i.e. affecting drug response) have higher

scores (mean  $\ell_C$  within protein) than variants categorized as pathogenic (Supplementary Tables 4.1, 4.2 and Figure 4.9). We speculate that this might be because amino acids that can be compensated would be characterized as “benign” whereas deleterious amino acids changes cannot be compensated by mutations in other proteins.

**Comparison to other Co-Evolutionary methods** There are several methodologies that can be used to predict putative interactions based on co-evolutionary theory. Nevertheless most methods are limited with respect to their applicability to GWAS-scale analyses:

*Phylogenetic tree similarity* can be used as a proxy for the co-evolution of interacting proteins. Computational methods use matrix alignment [191] which has demonstrated some degree of success. Unfortunately, such methods have two limiting factors: i) they require large (distinct) phylogenetic trees for each protein which are not be available for all proteins in the genome; and ii) solve an optimization problem requiring long computational times to match matrices (e.g. simulated annealing) for each putative pair or proteins.

*Correlation and Mutual information* based methods aim to detect changes on one of the interacting proteins that are compensated by mutations in the other [178, 92]. Although these methods are fast enough to be applicable to GWAS scale studies, they still have at least two limitations: i) they require large number of sequences in the multiple sequence alignment to overcome noise (as we mentioned large MSAs are not be available throughout the whole genome); and ii) there are affected by biases mainly caused by phylogenetic relationships and indirect correlations. There are methods based on mutual information that could perform some phylogenetic correction [67], nevertheless they still require a large number of sequences in the MSA and are known to be biased by allele frequencies [73] which might limit their applicability for GWAS studies (particularly on non-common variants).

*Global models* are designed to disentangle direct interactions from indirect ones. Several methods have been proposed which rely on: i) estimating parameters of Boltzmann distributions [138, 243], ii) mean field approximations of Boltzmann distributions [164], iii)

constrained optimizations for finding approximations of the inverses of large singular matrices [119], iv) marginalizing multidimensional extensions to mutual information [42]; or v) solving a Bayesian network model [30]. All these models are so computationally heavy that can only be applied to very small sets of proteins. Furthermore, in some of the respective papers the authors mention that it is computationally infeasible to apply them to a single pair of proteins if the length is over a some low number of amino acids (e.g. 60 [243] or 500 [164]). It is therefore not possible to apply any of these method to GWAS-scale studies at the moment.

Our method attempts to solve three of the main problems common to some of the aforementioned methods: i) the requirement of large number of sequences, ii) the phylogenetic bias, and iii) running time. These goals are achieved, at least partially, by using a well known Markov model of evolution.

**Comparison to other Epistatic GWAS methods** Many reasons have been given in this manuscript and elsewhere to indicate why detecting epistasis this is a very difficult task, with the most commonly cited one being the enormous number of statistical tests required, consequently having a significant reduction of statistical power and an increase in computational resources required. Thus methods based on exhaustive search can be computationally infeasible for all but very low order interactions analysis [53]. Their counterpart are *conditional search* methods [147] which are usually based on selecting the top single-marker GWAS results and then performing epistatic analysis on a small subset. Unfortunately these methods will ignore pure epistatic interactions and only detect marginal ones [147, 52]. Since there is no biological indication on whether complex traits have marginal or pure epistatic effects [60, 261, 147], it might not be safe to rely exclusively on these type of methods when performing an association study.

Stochastic search based methods [257] show great potential, but as far as we know have not yet produced any significant results most likely due to the small sample sizes used in the

respective publications. Some authors pointed out that some of most well known methods in this family may have difficulties with large number of samples [67].

Finally methods based machine learning have been proposed and applied with different degrees of success [129, 53, 147]. One of the main limitations of machine learning methods lies in the fact that the majority of them do not result in a statistical significance metric (such as p-value or Bayesian factor), thus researchers are often weary of conducting an expensive follow up studies based in results from machine learning algorithms. Another limitation is that many machine learning approaches do not allow for appropriate correction for population admixture and other cofactors.

The methodology we proposed in this paper is based on a well established statistical procedures (Logistic Regression) using standard corrections for known population cofactors (eigen-analysis) as well as other disease cofactors (such as age and sex) known to affect risk of type II diabetes. Our method performs an exhaustive search of second order interactions thus is capable of finding pure epistatic interactions pairs as well as marginal ones. Finally, we address power limitations by using co-evolutionary results from a well established Markov model of evolution and combining them with our association analysis by means of a Bayesian model. This has the advantage of not only being based on solid and well accepted theoretical grounds, but also being able to increase statistical power and to analyse large GWAS datasets that are becoming available now. Using distribution estimates (see figure 4.2), we calculate that our method can increase Bayes Factor between  $10^2$  and  $10^4$  when co-evolutionary information contributes to the total Bayes Factor (i.e. when  $\ell_C \geq 0$ ). This improvement is roughly equivalent to increasing the genome wide association p-value threshold by two orders of magnitude [95], (i.e. shifting the power curves in Figure 4.7 to the left). Thus the net effect of adding information from a co-evolutionary model is to reduce sample size requirements roughly by half, which is remarkable given the small amount of additional information being used by the model (only a multiple sequence alignment).

As a limitation, our methodology currently cannot analyse variants that are in complete

LD. There is evidence suggesting that nearby genes (in genomic coordinates) tend to have correlated expression levels and may to be in the same pathway or even interact [181]. Nevertheless it seems that complete LD would be a result of “selective sweeps”, in which an allele giving significant fitness advantage becomes more frequent so rapidly that there is little recombination. These selective sweeps seem to be rare [107], thus this limitation might not be encountered often in practice.

**Future work** We plan to extend our method to include context specific information by creating  $Q_2$  estimates for different protein domains, in order to obtain better estimates for well characterized protein interaction regions. Another line of work is to perform GWAS using kernel based statistics of multiple variants [251] thus allowing simultaneous analysis of nearby variants in a putative interaction hotspot. In this case the epistatic information would be used as a function modifying the kernel, instead of a Bayesian prior. It has also been suggested that positive selection might be used as an additional prior of epistatic interactions. Nevertheless a study comparing positive selection maps from nine different methods [5] shows that only 14% of the regions have been identified in more than one study. Although there seems to be a lack of concordance at the moment, using positive selection estimates would be an interesting venue to explore in the future.

## 4.7 Supplementary material

Significance (CLNSIG)	Count	Mean[ $\ell_C$ ]	Median[ $\ell_C$ ]
Benign (2)	272	34.1	26.2
Likely benign (3)	258	31.5	25.5
Likely pathogenic (4)	562	17.5	12.0
Pathogenic (5)	4206	16.9	11.7
Drug response (6)	18	32.6	22.1
Other (255)	10	20.0	11.3

Table 4.1: ClinVar categories have different distributions. Columns 3 and 4 show the mean and median  $\ell_C$  values calculated for each variant using the best (highest) within protein  $\ell_C$

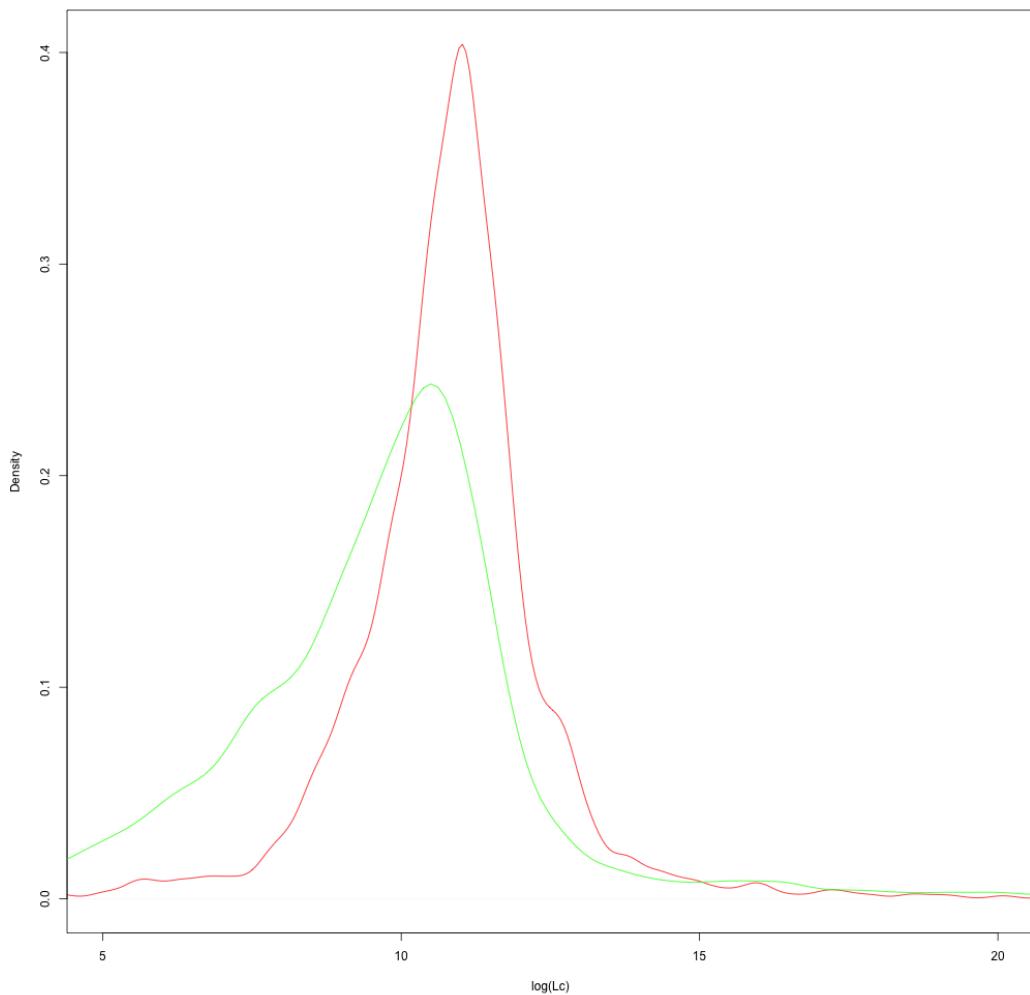


Figure 4.8: Distribution of  $\ell_C$  for interacting genes (red) and non-interacting genes (green) showing a small but statistically significant difference.

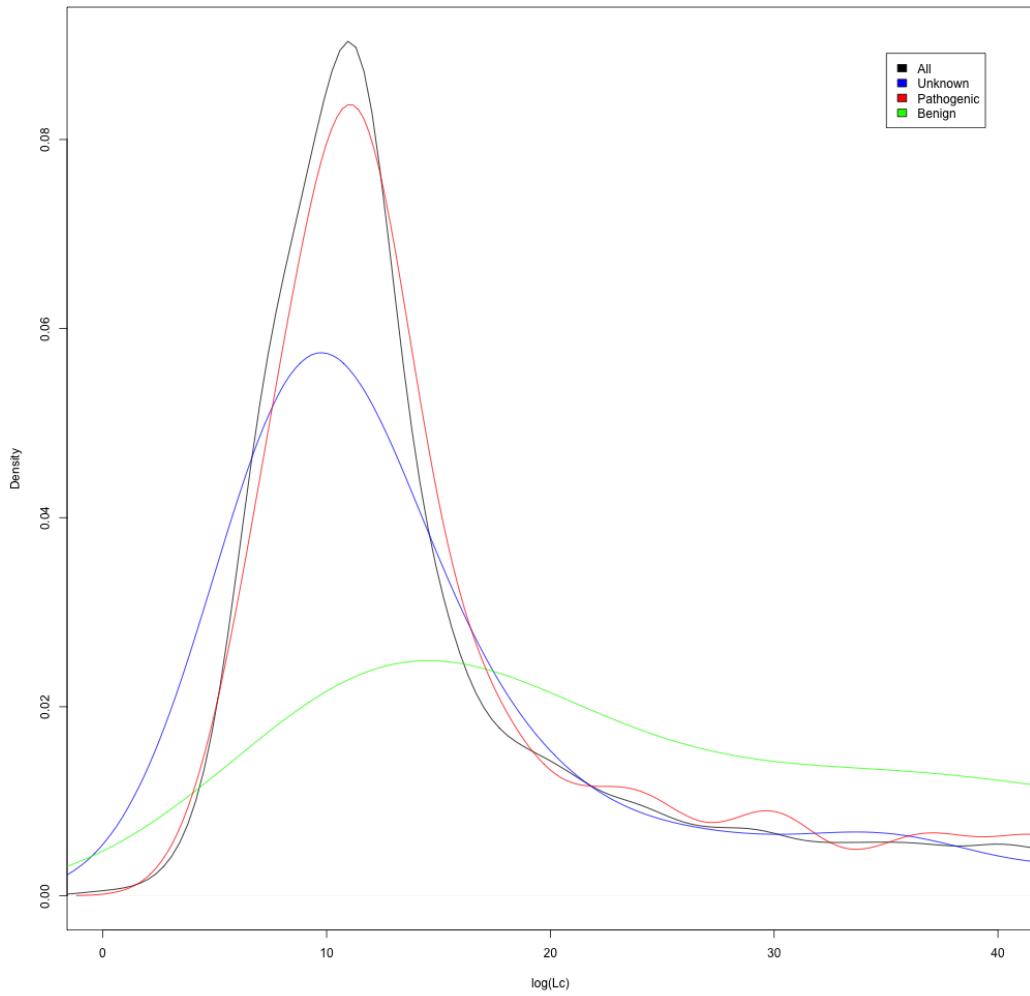


Figure 4.9: Distribution of  $\ell_C$  across different clinical categories from ClinVar database showing a clear separation on “*Benign*” variants

Project	$Mean[\ell_C]$	$Median[\ell_C]$
1000 Genomes	23.2	14.8
HGMD	19.8	12.1
ClinVar	18.6	11.9

Table 4.2: Overall distributions for 1000Genomes, HGMD and ClinVar. Columns 2 and 2 show the mean and median  $\ell_C$  values calculated for each variant using the best (highest) within protein  $\ell_C$ . Values are calculated using a random sub-sample of all variants in each project.

# Chapter 5

## Conclusions

### 5.1 Contributions

In this thesis I contributed to three steps involved in the analysis of human sequencing data and identifying the links between genetic variants and disease. Each step is characterized by very different types of problems:

- i) The first step is to reduce large amounts of information generated by high throughput experiments into a manageable summary. In our case, it involves reducing the raw sequencing information to a variant call set, but it could be any other features to be analysed (RNA expression, transcript structure, enrichment peaks, genome reference assembly, etc.). This is mainly done by mapping reads to a reference genome and then using variant call algorithms. This step is characterized by requiring fast parallel algorithms and usually, due to the amount of data involved, I/O can be one of the bottlenecks. Algorithms that work on “chunks of data” instead of the whole dataset are preferred, and in many cases exist, because working on disjoint data makes the problem easier to parallelize. Usually several stages of these highly specialized algorithms are combined into a “data analysis pipeline”. Programming data analysis pipelines is not trivial since it requires process coordination, robustness, scalability

and flexibility (data processing pipelines, particularly in research environments, tend to change often). Although data pipeline solutions are often available in the form of libraries, these libraries tend to make pipeline programming cumbersome or create new programming paradigms and thus introduce a steep learning curve. In Chapter 2, we address problems related to pipeline programming in a novel way by creating a new programming language, BDS, that simplifies the creation of robust, scalable and flexible data pipelines. Although the main rationale behind the development of BDS was managing our sequencing data pipelines, it is a flexible programming language that can be applied to many large data pipelines.

- ii) The second step in our data analysis consists of functional annotation, prioritization and filtering of genetic variants. The main concern in the annotation step is performing an adequate filtering of what should be considered relevant variants for our experiment. Until not long ago there were no publicly available packages for functional annotation of genomic variants, in chapter 3 we introduced SnpEff & SnpSift, two variant annotation solutions that quickly became widely adopted by the research community.
- iii) Finally, in Chapter 4, we analyse the problem of finding genetic links to complex disease. This is known to be a difficult problem affected by several hidden co-factors that bias the results (e.g. population structure). Furthermore there are limitations, evidenced by missing heritability, implying that genomic links to complex disease may not be found using traditional GWAS methodologies. We show that alternative models that combine higher level information, may help to boost statistical significance.
- iii.a) We proposed a new methodology for addressing a difficult problem: the detection of interacting genomic loci (epistasis) that affect disease risk. Our models combine genotype information and co-evolutionary evidence. We show that efficient algorithms make these studies computationally feasible, albeit using relatively large computational resources.

iii.b) We were involved in a major project on GWAS of type II diabetes using a cohort of multi-ethnic unrelated individuals which uncovered new genes linked to diabetes. We applied our epistatic GWAS models to data from this type II diabetes sequencing study of over 13,000 individuals finding suggestive evidence of interaction.

These three chapters (three steps) complete our journey from “raw data” to “biological insight” trying to find the genetic causes of complex disease.

## 5.2 Future work

Here we propose several improvements, extensions and future directions of work for each of the topics discussed in this thesis.

**BigDataScript** We are adding native support for new clusters and frameworks, such as LSF [114], Mesos [108], Kubertes [96] as well as a “*Generic cluster*” API which allows the user to customize BigDataScript for any cluster or framework by encapsulating task management via user defined scripts. On the language specification side, we are exploring ways to add functional constructs such as `map`, `apply`, `filter` as well as support for *map/reduce* and *scatter/gather* which are convenient ways to define some problems in data pipeline programming. Finally we, will incorporate user-defined data structures or a basic class mechanism (BDS currently supports maps and list).

**Variant annotations** In an effort coordinated with the developers of other annotations tools (such as ANNOVAR [238], ENSEMBLs Variant effect predictor -VEP- [162], JAnnovar [116], etc.) we are creating new annotation standard for VCF files. We are actively collaborating with the “*Global Alliance for Genomics and Health*” (GA4GH) to create a

new variant annotation specification & API definitions. We plan to extend SnpEff’s variant annotation capabilities to *haplotype-based* annotations, which means taking into account phasing information to calculate compound variant effects (e.g. phased SNPs affecting the same codon or compensating frame shifts within the same DNA strand). Finally, we are using information-theoretic analysis of splice sites from several species in order to improve splicing effect predictions.

**GWAS Epistasis** As future work, we’d like to evaluate the possibility of incorporating contextual information, such as protein domain, in order to build more specific co-evolutionary models. Other improvements include further optimization of logistic regression and Bayes factor algorithms since any improvement greatly reduces computational times. We also plan to use our methods on even larger type II diabetes cohorts that are currently being sequenced. Finally, we are evaluating the possibility of incorporating higher order interactions by clustering genes from our variant-pairs analysis and then evaluate them in a joint analysis.

### 5.3 Perspectives

Genomic research for complex disease is trending towards larger and larger cohorts in order to improve statistical power. Some years ago, projects involving hundreds to a thousand individuals were common. To put this in perspective, that is the population of a village, or a small town. Nowadays, projects like those lead by the T2D consortia sequence in the order of 20,000 people (i.e. the population of a large town). Projects are being drafted for sequencing over 100,000 individuals [193] (i.e. the population of a small city) and some institutions are foreseeing sequencing up to 1,000,000 samples per year within the next few years [194].

As a rule of the thumb, sequence data of a single whole genome requires  $\sim 800$  CPU hours of primary processing (i.e. read mapping and variant calling). For an institution

planning to process 1,000,000 genomes per year will require  $\sim$  800,000,000 CPU hours just for primary processing. In order to keep up with sequencing, data analysis should be also performed within the same time-frame, thus requiring  $\sim$  92,000 CPUs processing data continuously (an over optimistic estimate that assumes no hardware failures, no software failures and no programmed outages). Having tens to hundreds of thousands of CPUs constantly analysing data in production environments poses infrastructures challenges. Most academic environments currently use their own infrastructure (local clusters), an approach that may not be easy to scale further. For this reason a shift towards a cloud infrastructure is already being considered by some leading institutions (personal communications).

We developed BDS to help processing both the large datasets currently available, and also huge datasets that experts consider likely to become available in the near future. Even though BDS can currently handle typical analyses involving tens of thousands of CPUs, further scaling to hundreds of thousands or even millions of CPUs would require additional abstraction levels. Most notably, the current processing model assumes the existence of a file system which is used for retrieving input files, storing output results and and logging process status. We anticipate that this model can break down on cloud based pipelines running over hundreds of thousands of CPUs. Typically cloud based environments use the concept of object storage (also called buckets) instead of file systems. We think that the two models (file system and object storage) can be abstracted away in a new unified model enabling the user write even more portable pipelines and letting BDS take care of transparently transferring data to and from the object storage system. This approach has the benefit of also enabling users to transparently add data locality optimizations by moving the processes close to the data instead of the traditional approach of moving the data to the process, with various degrees of data locality optimizations ranging from multi-datacenter processing to rack-aware file systems.

The quest for ever bigger sample sizes shows how elusive the genetic causes of complex diseases are. It might be true that huge sample sizes are needed to uncover risk loci, but

perhaps one of the reasons why traditional GWAS studies have not found as many associations as expected is that we they are looking at the wrong place by not routinely taking into account other types of disease variants (e.g. InDels or CNVs) or models for interacting variants (epistasis).

In the context of large cohort studies, variant annotation improvements would greatly benefit the outcome. Compared to the previous problem of processing large datasets, variant annotation is challenging not because of the computational challenges but rather due to restricted biological knowledge. Advanced variant effects models could be developed with help of systematic studies. For instance, a systematic analysis of loss of function and nonsense mediated decay variants would entail creating all possible stop gained mutations in one or more genes and analysing the protein output in each case (obviously this is an ambitious and challenging project, but so were other projects like 1KG [47], GTEx [151] and ENCODE [49], just to mention a few). Lower impact variants, such as non-synonymous variants, pose even further challenges since there is no consensus on how to measure partial protein gain or loss of function (e.g. in a protein affected by a non-synonymous variant, interaction efficiency with protein X is degraded by 50% whereas interaction with protein Y is improved 20%). Such analyses, which are beyond the current state of technology, could only be feasible by supporting long term technology development projects.

Finally, we should keep in mind that the ultimate goal of complex trait research is to have an impact on human health. This implies that research results should be readily available for translational medicine to effectively use of them. Scientific journals date from 1665 [131] and understandably has some shortcomings in the era of translational medicine. An enormous effort is required to read papers, curate them, and translate their results into meaningful coherent data. In the private sector, companies that embark on this costly curation process regard the resulting curated databases as a competitive advantage and are unwilling to share them, creating the well known silo effect which leads to several fragmented isolated curation projects with various degrees of success and different qualities. On the other hand, there are

few incentives in academic environments to create curated databases or to maintain them after the paper is published and the students leave their labs, resulting on a plethora of outdated databases with different curation standards. A refreshing approach adopted by the ClinGen project attempts to create long term, well curated, high quality, clinically relevant database/s. Obviously it would be better to have researchers contribute directly to these efforts thus significantly reducing the curation burden, but currently there are no incentives for researchers to do so. If we want to make more effective use of research results in clinical environments, research agencies should incentivize (or even require) investigators to publish genomic results in ClinGen and other similar systematic long term efforts that might appear in the future.

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