Next generation genomics tools for wheat improvement

Ricardo Humberto Ramírez-González

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Abstract

In recent years the amount of genomic resources of wheat has increased to the point where manual analysis is unfeasible. The aim of this PhD was to develop bioinformatics tools that help answer biological questions relevant to research and breeding by addressing the complexities associated with the wheat genome. I took advantage of resources which became publicly available as the analyses were carried out and I developed new approaches, strategies and tools to help accelerate wheat research. Chapter 1 reviews the genomic resources used for the thesis, placing them in historic context with the work and analyses performed. Chapter 2 describes the development of PolyMarker, a bioinformatics pipeline to design genome-specific primers in a timely and effective manner. Examples of different applications of PolyMarker are also included. Chapter 3 describes the analysis of an F_2 population to generate a genetic map for *Yr15*, a gene that provides resistance to yellow rust. The SNP calling was done from bulked segregating samples, sequenced with RNA-Seq as a method of reduced representation. Chapter 4 describes expVIP, a tool to integrate RNA-Seq experiments in a relational database. Data from different studies can be visualised simultaneously, enabling comparisons between them. Lastly, in Chapter 5 all the data types used for the analysis on each of the previous chapters is integrated into a relational database. The discussion further explores how genetic maps, SNP markers, novel SNPs, gene annotations, gene assemblies and gene expression can be used simultaneously in research and breeding programs. All the tools and pipelines described in this thesis are open source and are available on: `https://github.com/homonecloco`.

Contents

List of Figures

List of Tables

Listings

"We passed through forests of fire,
forded rivers of light and forged dark seas
and mountains of snow and ice.
Each crossing took us thousands of years,
though it seemed no more than the blink of an eye."

ORHAN PAMUK, MY NAME IS RED

Acknowledgements

This PhD could not have been possible with the support of my family, friends and colleagues.

First, I would like to thank my supervisors, Mario Caccamo and Cristobal Uauy for their unconditional support, regardless the circumstances. They had been good at nurturing my curiosity with all this "just one week projects" that end up thesis chapters, peer review articles or just a lot of fun in the process of learning how not to do things. I must thank them also for their trust on me, even when I haven't been sure of my own capacity.

I would like to thank my family for their unconditional support. On the distance, we remain together.

I am extremely grateful to Esther, Luca and Evanthia who showed a lot of filotimo when proofreading and giving feedback in very short notice, without even being asked.

If I am sure I am missing some old an new friends that had given light in this four years, to all of you: Thanks.

Finally, I want to dedicate this thesis to the loving memory of my grandparents who left this world just a few months before giving them the joy witnessing the completion of this PhD.

Chapter 1

Introduction

1.1 Wheat Genetics

1.1.1 Polyploidy and Wheat

A polyploid species contains more than one set of related genomes, that may come from a chromosomal duplication (autopolyploid) or from an hybridisation with a related species (allopolyploid; Shewry 2009). *Triticum aestivum* (bread wheat) has gone through an speciation event and two major hybridisation events. Initially, a common progenitor evolved into two different species around 7 million years ago to form the A and B genomes, whose closest known relatives today are *Triticum urartu* and *Aegilops speltoides* (Dubcovsky and Dvorak, 2007), respectively. Around 5.5 million years ago, these two lineages hybridised though homoploid hybrid speciation to give rise to the D genome progenitor *Ae. tauschii* (Marcussen et al., 2014; El Baidouri et al., 2016). Homoploid hybrid speciation refers to the event which occurs when a fertile lineage, in this case *Ae. tauschii*, results from hybridisation between two distinct species (*T. urartu* and *Ae. speltoides*), without a change in ploidy level (Gross, 2012). Then, less than 800 thousand years ago the ancient A and B genome species hybridised and formed a tetraploid wheat, *Triticum turgidum* ssp. *dicoccoides* (wild emmer). A final event occurred less than 400 thousand years ago, when emmer wheat hybridised with *Ae. tauschii*, leading to bread wheat (Figure 1.1.1, Marcussen et al. 2014). Wild emmer tetraploid wheat was later domesticated to give rise to pasta wheat (Dubcovsky and Dvorak, 2007).

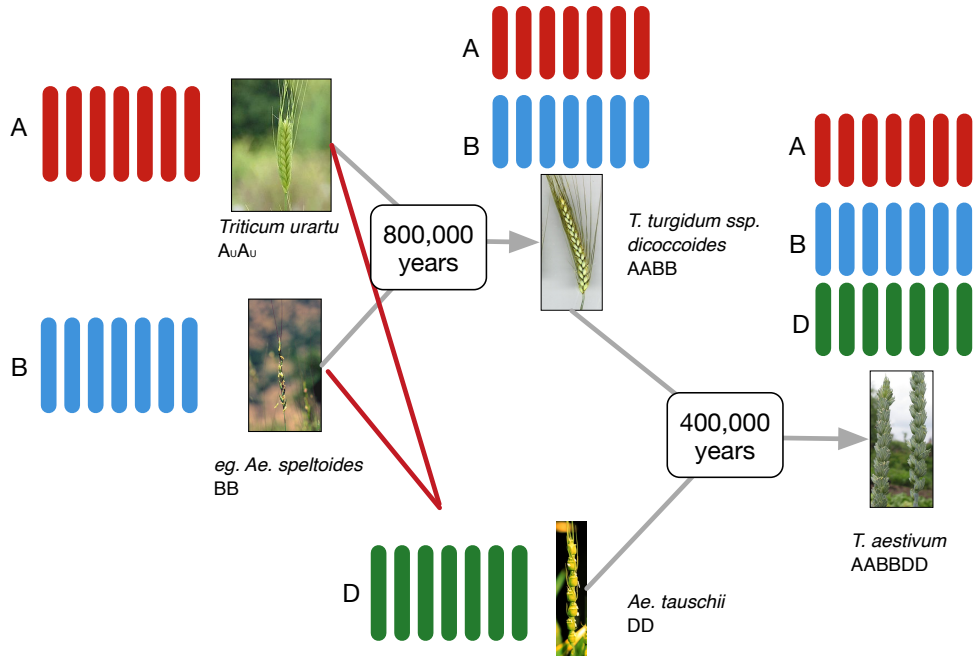


Figure 1.1: Hibridisations that led to bread wheat *T. aestivum*. Seven coloured chromosomes are drawn alongside each species to depict ploidy level and genome constitution.

Because bread wheat contains three related, yet independent copies of its genome, the expectation is that it has three copies of each genes, which are referred to as homoeologs. The actual values are close to this expectation with some variations depending on specific genes. Overall, the first global analysis by Brenchley et al. (2012) showed that the hexaploid wheat genome had a gene ratio of between 2.5:1 and 2.7:1 compared to the D genome.

1.1.2 Experimental lines

In crops, genetic research usually consists on crossing individuals and study the traits of the next generation. The progeny of a cross is called population. Groups of seeds with the same genetic background are called lines. Plants with the same homozygous background are pure lines. To study particular locus experimental lines are developed, the following list describe the most common (Van Ooijen and Jansen, 2013b):

F_1 The first generation of the cross between two plants. If the progenitors are homozygous and different, all the lines in the resulting population are heterozygous at any given locus.

F_2 populations come from a single heterozygous F_1 plant or population that is crossed to itself. The progeny is segregating (Figure 1.2a) with homozygous and heterozygous individuals. This type of population is used for the experiments in Chapter 3 and it is described in more detail in Section 3.1.1.

Back-cross (BC) lines are used to fix a trait on a genetic background. The process starts from a plant in the F_1 with the desired genotype or phenotype. This plant is crossed again ("back-crossed") to a plant from the line used as background (P_1; known as the recurrent parent). The progeny are called Back Cross 1 (BC1; Figure 1.2b). A plant from the BC1 with the desired genotype is selected and crossed again to P_1. The process can be repeated, and with each cross the region linked to the target locus is narrowed and the background becomes more similar to P_1.

Near Isogenic Lines (NILs) After BC6, a line is considered Near Isogenic (Stam and Zeven, 1981). At this level of back crossing most of the genetic material is the same as P_1, except for the region linked to the trait which has been actively selected during the crossing scheme. In most cases sibling lines are selected so that pairs of NILs are used either carrying the desire trait or not.

Recombinant Inbred Lines (RILs) are used to produce homozygous lines from an F_2 population. Each plant in the population is self-crossed. A single plant is usually selected and self-pollinated again in a process usually called "single seed descent". After several iterations the line is considered homozygous. 1.2c).

Doubled Haploid (DH) lines are an alternative technique to produce homozygous lines. The individuals on the F_1 population are crossed to a different plant (ie maize crossed to produce wheat DH) to simulate pollination (Reviewed in Niu et al. 2014) . Under natural conditions, the gametes would be aborted, therefore the embryos are rescued and treated with colchicine to induce a genome duplication. Since the duplication comes from a single gamete, the resulting plant is homozygous (Figure 1.2d). This process is quicker than the production of homozygous RIL, but is more technically demanding and expensive.

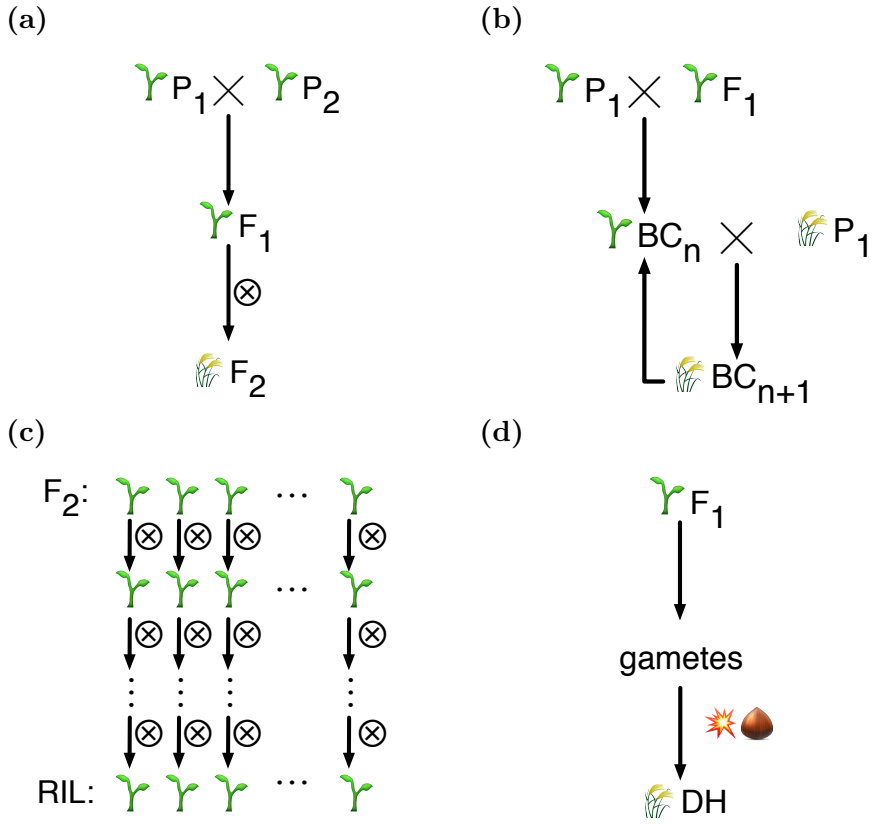


Figure 1.2: Types of experimental lines. represent populations and individual plants. ($\times$ represent a cross between lines. ($\otimes$) represent self-pollination. Ellipsis (...) represent repetition. represent a treatment to double the chromosomes from the gamete. (a) F_2 population. (b) Back Cross population. (c) Recombinant Inbred Lines (RIL). (d) Doubled Haploid (DH).

1.2 Sequencing

Over the past 10 years there have been multiple developments in sequencing technology that have revolutionised many aspects of modern science. Details of the different methods and their history have been reviewed extensively (Goodwin et al., 2016) and are outside the scope of this thesis. However, more details will be provided on the Illumina system since this technology is most heavily used in the thesis.

1.2.1 DNA sequencing with Illumina

In the Illumina sequencer, the DNA library is loaded into a flow cell, where the fragments are captured by their adaptors. Each fragment is amplified into clonal clusters, resulting in multiple copies of the sequence bounded to the surface in close proximity. Then, the sequencer detects the fluorescent dye-labelled oligonucleotides that are added one by one to the bound sequences by taking multiple images. This process can be repeated for each pair of the DNA. copies of the same sequence are clustered together, it is possible to analyse the images and detect which base is added to each cluster, determining multiple sequences with high fidelity, and exporting them as a FastQ file (Goodwin et al., 2016; Cresko Lab, 2015; Illumina Inc).

According to the objectives of the experiment and the quality and volume of the available DNA, the library can be prepared on fragments of different sizes, the classification of the available sequencing for the fragments is the following Myllykangas et al. (2012); Metzker (2010); Shendure and Ji (2008); Hutchison (2007):

Single end. The DNA fragments are sequenced in from the 5'-end. This can be used when long DNA fragments can't be produced due to the quality of the sample(i.e. ancient DNA), or when the target are small molecules (i.e. expression of microRNAs).

Paired end. As in the single end sequencing, the 5'-end of a the fragments are sequenced. However, after sequencing one strand, the process is repeated with the reverse complement. This allows to to get more sequence for each fragment and an indication that both fragments are close.

Overlapping Read Pairs. A variation to paired end sequencing, where the size of the fragment is shorter than two times the read length. This allow an alignment between the two reads to get an longer contiguous read with the limitations of the instrument.

Mate pairs. Used to get reads separated at distances between 1kbp and 5kbp. To achieve this, the molecule is circularised and the point were the two ends of the fragment were joint a biotin marker is inserted. Then, the molecule is fragmented again and the fragments containing the biotin are sequenced in the same fashion that read pairs. The resulting reads have the same orientation.

1.2.2 RNA-Seq

During transcription, a DNA sequence is transcribed into the primary RNA transcript (pre-mRNA) by the RNA polymerase. The RNA polymerase, and associated transcription factor, binds to promoter DNA and creates an RNA complementary copy. Depending on multiple factors, including gene regulation, this process can be repeated to create multiple copies of the sequence(Alberts et al., 2014). The pre-mRNA suffers various modifications like splicing and capping. In a gene, there are intragenic regions, called introns, that are removed from the pre-mRNA, while the remaining sequences, called exons, become part of the mature mRNA. Splicing is catalysed by the spliceosome, which recognises the sequences of the splice donor, branch and acceptor sites in the intron, cuts the intron and pastes the remaining exons. In some cases, different introns and exons can be included or excluded, creating alternative splicings of the RNA sequence (Alberts et al., 2014). Then, the 5' and 3' ends of the RNA sequence are modified to generate a mature messenger RNA (mRNA). A 7-methylguanosine cap is added to the 5' end. The 3' end is cleaved and polyadenidated, acquiring a poly(A) tail of approximately 200 adenines (Alberts et al., 2014). The result of this process is a mature mRNA, which forms part of the transcriptome.

Both DNA and RNA can be sequenced using available Next Generation Sequencing (NGS) technologies. The preferred starting template will depend on several factors including the biological question at hand, the size of the genome and gene space, the resources available, etc. In the case of wheat, complete representation of DNA is impractical given

the genome size (Borrill et al., 2015). Therefore reduced representation methods constitute an important alternative (Figure 1.3). Among them, we used exome capture and RNA-Seq during the course of this PhD. However, my main focus was on the use of RNA-Seq and therefore I detail more on this methodology below. Originally, sequencers where designed with DNA in mind, so that analysing RNA requires converting the transcriptome into a cDNA library (Wang et al., 2009). To begin with, RNA is first isolated, purified and enriched for mature mRNA. For some sequencers, the resulting mRNAs need to be fragmented to improve sequence coverage, as current sequencers can only read sequences shorter than the transcripts. Once the quality of the RNA has been verified, it is converted into cDNA and adaptor sequences are added. First, the RNA strand is copied into first strand cDNA using the reverse transcriptase, as traditional polymerases cannot convert from RNA to DNA (Alberts et al., 2014). Reverse transcriptase, as other polymerase, requires a primer annealed to begin the polymerisation, so random primer are used to avoid 3' bias and improve coverage. To obtain the second strand, RNase is used to cleave the original RNA. The remaining fragments serve as primers for DNA polymerase I, creating a double-stranded complementary DNA. The cDNA fragments then go through an end repair process, the addition of a single 'A' base, and then ligation of the adaptors, which will be used by the sequencer to read the sequence. The products are then purified, to remove artefact and their quality is verified. The remaining sequences are enriched using the polymerase chain reaction (PCR), which can generate thousands to millions of copies of a particular DNA sequence (Illumina Inc). The resulting cDNA library is sequenced using available high throughput sequencing technologies.

1.3 Sequence analysis

From the computational point of view, the problem can be viewed as a string matching. The Smith-Waterman (Smith and Waterman, 1981) and Needleman-Wunsch (Needleman and Wunsch, 1970) algorithms are the gold standard interns of accuracy looking for similarity between sequences. However, the execution time for both of them is prohibitive to run in massive databases. The algorithm execution time is O(mn), as it requires calculating a matrix of size $m \times n$ where m is the target sequence

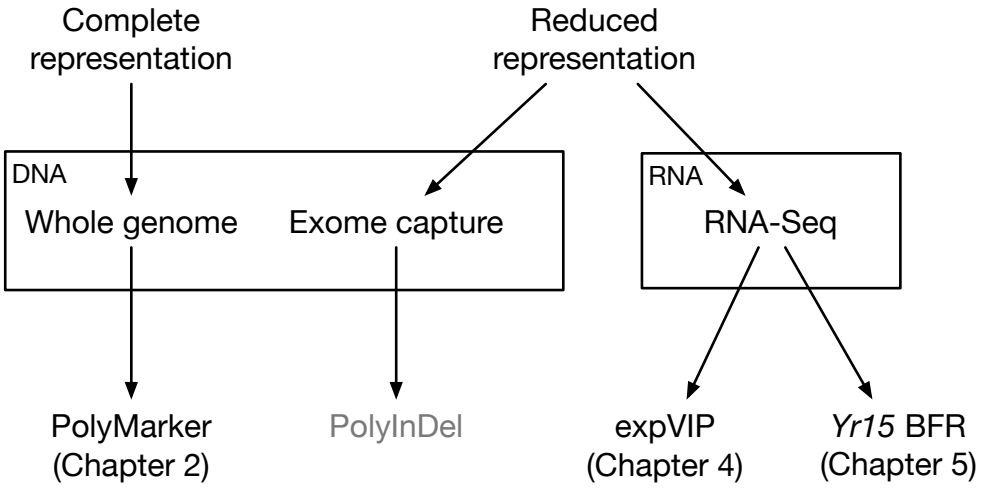


Figure 1.3: General types of sequencing ant their relationship with this PhD. PolyInDel is a project still in progress, not discussed in this Thesis.

and n is the query sequence. To scale this to a manageable problem algorithms like BLAST, BLAT and exonerate index the references to make the search faster. This alignments tools are useful for long stretches of DNA (like cDNA or contigs; Altschul et al. 1990).

BLAST requires to index the sequences used in the database. The heristic search looks for occurances of patterns of the target sequence in the index and the alignment is then extended doing a local alignment (Altschul et al., 1990). When the query sequence and sequence within the database have a high sequence similarity and length coverage this is defined as a 'hit'. The significant hits are generally termed as those that have protein sequence comparisons above 75% sequence similarity and high coverage.

BLAT Blast-Like Alignment Tool, is much faster than BLAST but less sensitive as it does a k-mer indexing of the database instead of a linear search, which means it finds seeds quicker. The way that BLAT performs quick analyses is by computing an "index of all non-overlapping K-mers in the genome". It is important to note that BLAT is less effective for sequences with less than 90% sequence identity (Kent, 2002). This level of identity is enough to find the homoeologous genes in wheat, as they have an identity over 92% between chromosomes (Krasileva et al., 2013)

Exonerate Is another alignment program that works as a faster alternative to exhaustive sequence alignment methodologies, by implementing bounded sparse dynamic programming (BSDP). It performs pairwise sequence comparisons, doing so by using various alignment models, exhaustive dynamic programming or different heuristic algorithms (Slater and Birney, 2005). Exonerate is able to model intron-exon junctions when doing the alignment.

When analysing high-throughput sequencing, having millions of short sequences make unfeasible to try to align the data to every possible reference. However, one can take advantage of the fact that you know which organism you are looking for and, if available, use a genomic reference. For this, tools like MAQ, BWA, Bowtie, among others, provide indexed search. Once the reads are aligned, to a reference the downstream analysis will depend on the biological question being asked and the type of sequencing carried on. Fortunately, most of the Short-Read sequence alignment produce similar outputs and the SAM format is becoming a *de facto* standard. This is allowing to make more a modular downstream analysis where you can test different aligners with different settings and pick the algorithm that better fits your experiment (Liu and Schmidt, 2012; Li and Durbin, 2009; Li et al., 2009).

IUPAC Ambiguity Codes

To represent polymorphisms in a single sequence the IUPAC has developed an representation of a single character combining more than one possible nucleotide. This table is used in Chapters 2 and 3 to represent Single Nucleotide Polymorphisms (SNPs).

Table 1.1: IUPAC ambiguity codes

IUPAC Code	Meaning	Complement
A	A	T
C	C	G
G	G	C
T/U	T	A
M	A or C	K
R	A or G	Y
W	A or T	W
S	C or G	S
Y	C or T	R
K	G or T	M
V	A or C or G	B
H	A or C or T	D
D	A or G or T	H
B	C or G or T	V
N	G or A or T or C	N

1.3.1 RNA-Seq

One way to narrow down which genes are involved in certain trait or response to the environment is to focus on studying only the expressed genes. One of the techniques involving NGS is RNA-Seq (See Section 1.2.2). Depending on how much *a priori* information of the analysed organism is available different bioinformatic approaches can be used.

Transcriptome alignment The reads are aligned to a database of known cDNA. Ideally, alternative splicing sequences are available, so a simple alignment should work (i.e. BWA, bowtie).

Genomic alignment The reads are aligned to the genome. The splice junctions, introns and axons need to be accounted, so simple alignment doesn't work. Regular alignments are used, but the reads may be trimmed at fixed sizes to allow discontinuous alignments using regular tools (i.e. Stampy, Tophat/cufflinks)

***De Novo* transcriptome assembly** If a reference of the organism is not available, it is possible to generate a draft transcriptome with the RNA-Seq reads with traditional assemblers (velvet, abyss) or with specialised assembler tools like Trinity.

Once you have the alignments it is possible to evaluate the relative expression of the genes in the sample calculating the Reads Per Kilobase of transcript per Million of mapped reads (RPKM) or the Transcripts per Million of mapped reads (TPM). This normalises the expression by the amount of sequenced data and can be used to find which genes change in expression volume across different samples.

1.4 Wheat genomics

During the course of this PhD, several resources were released in the wheat community. A timeline of the release of each one of those resource is in Figure 1.4.

1.4.1 Genomic sequence

The length of the genomic references available for wheat has been increasing year by year. During my PhD the following genomic references where published.

454 Liverpool. A whole genome shotgun (WGS) sequencing project for Chinese Spring (CS) done in 454. The average coverage was around 5x. Assembling the reads produced an assembly with shorter contigs than the original reads. Hence, it was released as raw reads (Brenchley et al., 2012). Despite not being a proper reference genome, the reads where enough to find variations across the genomes and was used to designed genome specific primers before the IWGSC reference was published.

IWGSC CSS. The International Wheat Genome Sequencing Consortium (IWGSC) is able to extract DNA from a single chromosome arm, using a method called flow sorting. DNA for each sample was sequenced with Illumina to a coverage of at least 60x and it was assembled (Mayer et al., 2014). The assembly is quite fragmented partly because the assembler not being able to cope with repetitive

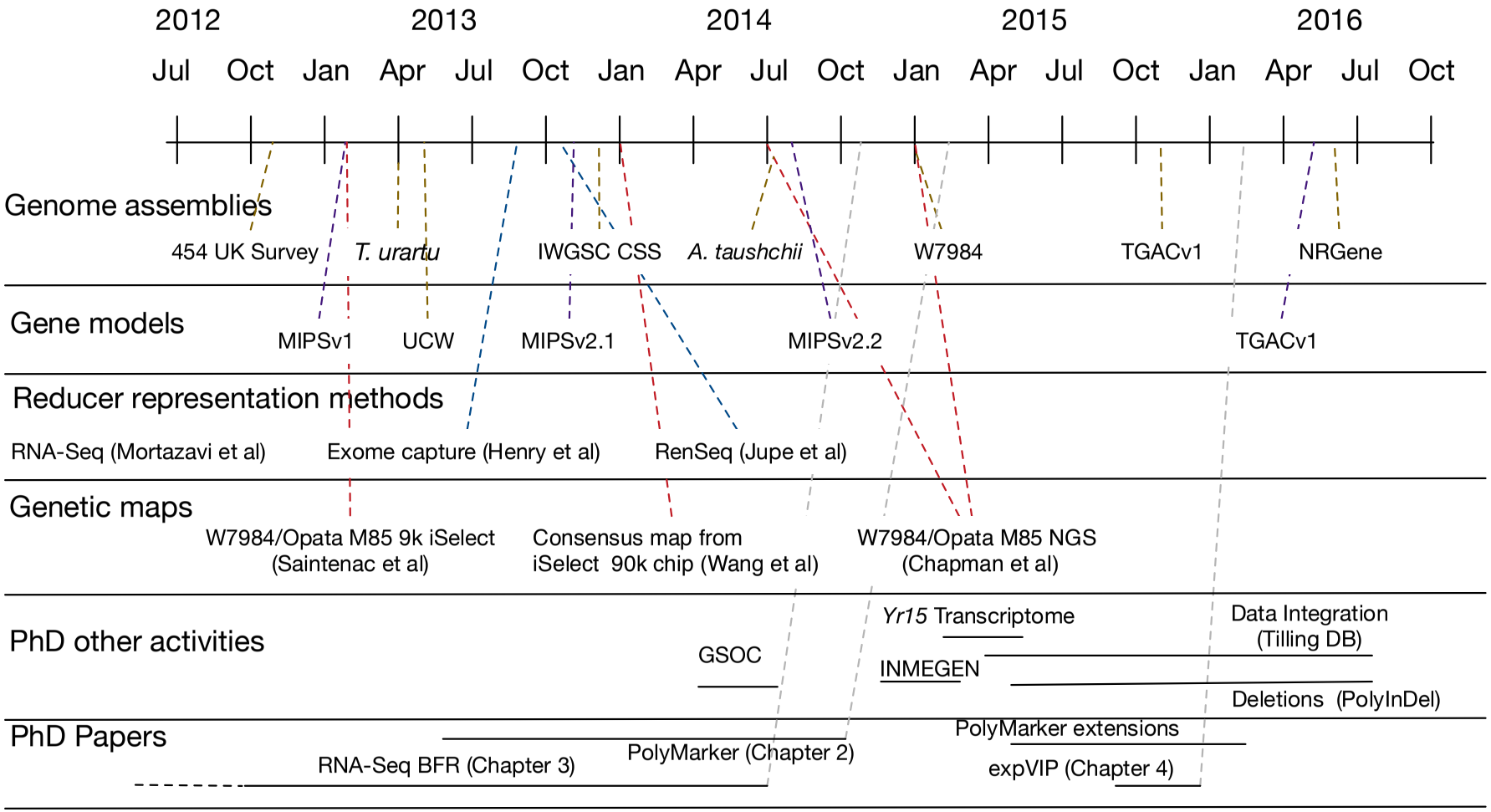


Figure 1.4: Timeline of the projects carried on during this PhD and the wheat resources that were released in the same time period.

regions, and partly because the flow sorting reduces the quality of DNA, preventing the preparation of long fragments.

Chapman. A WGS project, done with Illumina. Instead of sequencing CS, a synthetic wheat was used. The scaffolds are longer than the Chinese Spring Chromosome arm survey sequence (CSS) assembly, but the project was not annotated. It was used mainly to develop markers in a mapping population between synthetic (W7984) and non-synthetic (Opata) wheat lines (Chapman et al., 2015).

TGACv1 A WGS originally proposed as an improvement to the CSS assembly. The scaffolds are longer than in Chapman and it uses the contigs from CSS to assign the chromosome arm corresponding to each contig. An annotation for this assembly is available (Clavijo et al., 2016).

1.4.2 Gene models

For this thesis, a set of gene models correspond to the coding sequence of a gene. The following sets of gene models are used in this thesis.

UniGenes. This is an effort of the National Center for Biotechnology Information (NCBI) to unify several ESTs deposited on their databases, per species. The set is generated automatically by aligning all the ESTs to each other and clustering them by identity. The longest EST is selected as the canonical representation of the gene (Pontius et al., 2002). Because this approach is not aware of the different genomes, and the set of ESTs may not include them, the algorithm collapses homoeologous genes. This needs to be taken in account when doing down stream analysis.

UCW gene models. This set of gene models come for tetraploid wheat come from a *de novo* assembly of *T. turgidum* (AABB) a *T. urartu*. In both cases RNA-Seq was assembled with several parameters and the resulting assemblies where merged. To separate homoeologues genes that were assembled as a single gene, the reads were aligned to the assembly. Then, the phasing of the reads was used to separate the gene in the two homoeologues (Krasileva et al., 2013). This gene models are useful for tetraploid wheat. When using this reference

with hexaploid wheat, care must be taken of reads coming from the D genome that will map to one of the alternative homoeologues.

Genome annotation IWGSC. To complement the CSS genome assembly, the genome was annotated using related grasses (*Brachypodium distachyon*, *Oryza sativa*, *Sorghum bicolor*, and *Hordeum vulgare*. The annotation was supported by RNA-Seq data from five samples from different tissues at three developmental stages (Mayer et al., 2014). This was the first genome-scale annotation effort including the three genomes independently. The main caveat of this annotation is that due the relatively small size of the scaffolds used as reference, several genes are split in two different gene models.

Genome annotation TGACv1. This is the companion annotation for the TGACv1 assembly. The annotation was produced with a novel approach, by merging four alternative assemblies from three RNA-Seq datasets and long reads from PacBio sequencing (Venturini et al., 2016). The polished assemblies and full length cDNA alignments were used together with protein alignments as basis for evidence-based *ab initio* predictions with Augustus. This gene models were made public during the last year of this PhD, hence they were not used for the analysis. However the tools described in Chapters 2 and 4 can work with the updated gene models.

1.4.3 Genetic markers and maps

The development of high resolution genetic maps had been benefited by technologies like SNP arrays and NGS. SNP arrays allow the genotyping of thousands of alleles from a single plant. Likewise, low coverage sequencing can be used with the support of a good reference to do genotyping. Both techniques had been used recently to produce the following genetic maps:

90k iSelect chip The marker sequences used for the design of this array were defined from 81,597 SNPs coming from 19 hexaploid and 18 tetraploid wheat accessions and, a an aggregation of previously published SNPs. The wheat accession where sequenced with RNA-Seq and the reads where used to detect polymorphisms. The se-

quence flanking the SNPs come may include the intron-exon junction. 46,977 of the SNPs were genetically mapped with a combination of eight mapping populations (Wang et al., 2014).

820k Axiom chip The SNP discovery for this array was done using exome capture from 43 bread wheat and relative species. Three mapping populations (Avalon × Cadenza, W7984 × Opata and Savannah × Rialto) were used to produce a consensus map with 56,505 markers (Allen et al., 2016; Winfield et al., 2016). This points out that a higher number of markers does not necessarily means a higher resolution in the genetic map, the main constrain are the mapping populations.

Popseq/Chapman The SNPs came from a mapping population of 90 individuals, all consisting of DH lines from a cross W7984 × Opata. Those DH lines were lightly sequenced, with a coverage less than 2×. Because of the high density of the SNPs, the haplotypes could be inferred. The total number of SNP is over 24 million, but the minimal set of markers 112,687 for the genetic map. The same population was used for the original CSS assembly (Mayer et al., 2014) and the assembly from Chapman et al. (2015).

1.5 Aim and objectives

The main aim of this PhD is to develop bioinformatics tools that help answer biological questions of importance by addressing the complexities associated with the wheat genome, such as its size and polyploid nature. These tools can only be effective if they are designed within a biological context and as such I have worked alongside experimental biologists over the past four years. The strategies and resources developed in this PhD take advantage of the latest developments in wheat research and I incorporated these as they were made public (Figure 1.4). The topics covered by this thesis, their relationship and, the chapter where they are used are shown in Figure 1.5.

As my aim was to develop new approaches, strategies and tools in polyploid wheat, I have listed a series of objectives, rather than specific hypotheses, which I tried to address over the course of this PhD.

1. Simplify and automate the design of primers for marker assisted selection in polyploid wheat (Chapter 2).

2. Develop a pipeline that can be used to find genetic markers for breeding, using bulked segregant analysis and reduced representation sequencing (Chapter 3).

3. Develop a platform to integrate and visualise RNA-seq expression experiments (Chapter 4)

4. Establish an overall framework to integrate the different resources developed in this PhD (Chapter 5).

All the pipelines and data produced by this project are publicly available. Towards this end I have also strived to publish this research in open access journals. Peer reviewed publications stemming from these research chapters are listed below:

- Chapter 2 (PolyMarker)
  - **Ramirez-Gonzalez RH**, Uauy C, Caccamo M (2015) PolyMarker: a fast polyploid primer design pipeline. Bioinformatics, doi:10.1093/bioinformatics/btv069 (corresponding author)
  - King R, Bird N, **Ramirez-Gonzalez RH**, Coghill JA, Patil A, Hassani-Pak K, Uauy C, Phillips AL (2015) Mutation scanning in wheat by exon capture and next-generation sequencing. PlosOne. 10 (9), e0137549
  - Hubbard A, Lewis CM, Yoshida K, **Ramirez-Gonzalez RH**, de Vallavieille-Pope C, Thomas J, Kamoun S, Bayles R, Uauy C, Saunders DGO (2015) Field pathogenomics reveals the emergence of a diverse wheat yellow rust population. Genome Biology 16:23
- Chapter 3 (Bulked segregant mapping)
  - **Ramirez-Gonzalez RH**, Segovia V, Bird N, Fenwick P, Holdgate S, Berry S, Jack P, Caccamo M, Uauy C (2014) RNA-Seq bulked segregant analysis enables the identification of high-resolution genetic markers for breeding in hexaploid wheat Plant Biotechnology Journal 13:613-624
  - **Ramirez-Gonzalez RH**, Segovia V, Bird N, Caccamo M, Uauy C (2015) Next Generation Sequencing Enabled Genetics in Hexaploid Wheat. in Advances in Wheat Genetics: From Genome to Field eds Ogihara Y, Takumi S, Handa H, pg 201-209
- Chapter 4 (expVIP)
  - Borrill P, **Ramirez-Gonzalez RH**, Uauy C. 2016. expVIP: a customisable RNA-Seq data analysis and visualisation platform. Plant Physiology 170:2172 (joint first author)

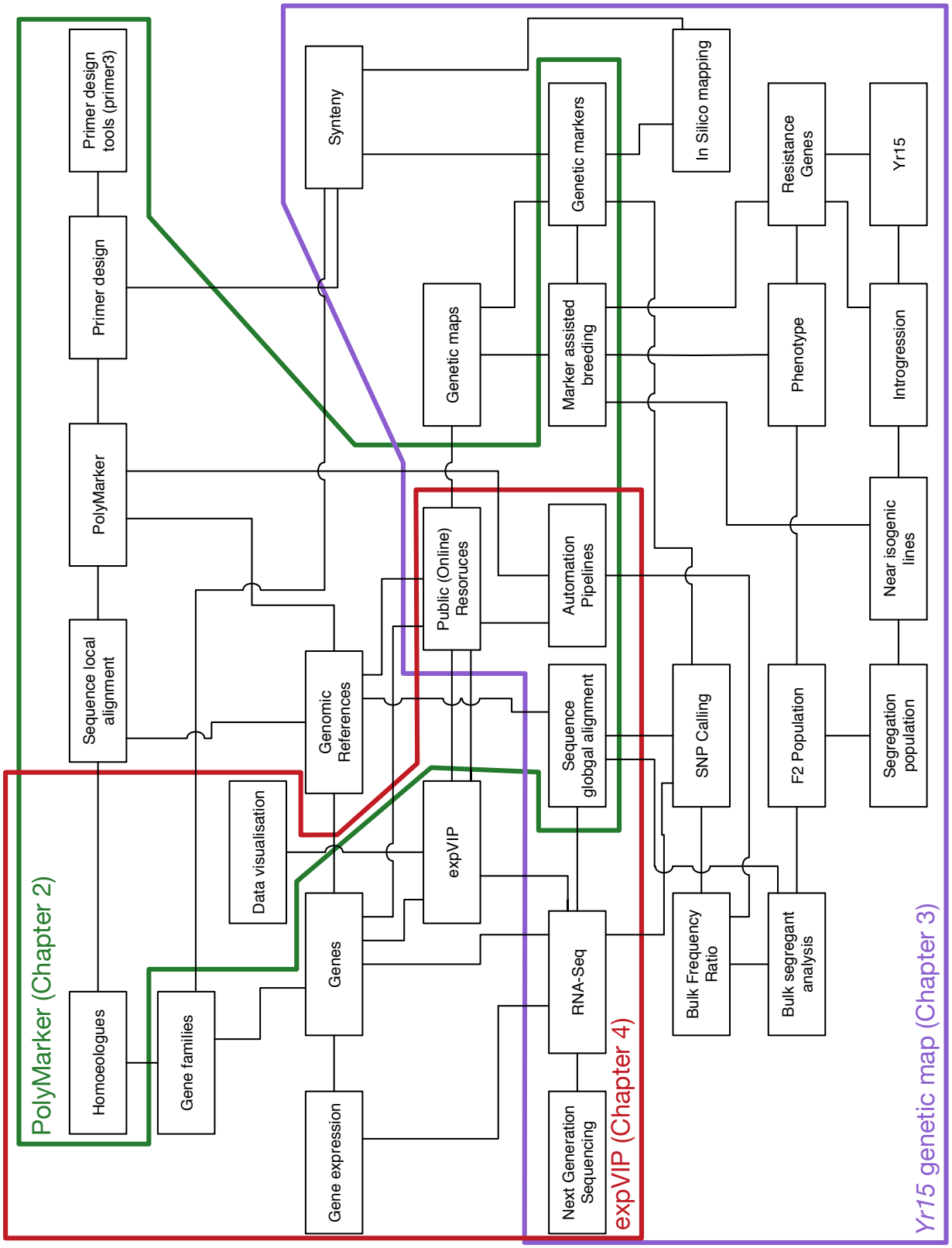


Figure 1.5: Relationships between topics of this Thesis. The lines represent connections between topics. Each coloured box represent a Chapter: Green for primer design in polyploid wheat (Chapter 2); Purple for the use of Next Generation Sequencing to produce a genetic map for *Yr15* (Chapter 3) and; Red for expVIP, an expression browser for Polyploids (Chapter 4). The intersection between all the chapter includes sequence alignment, the design of pipelines to automate the analysis and, making the resource publicly available.

Chapter 2

PolyMarker: A fast polyploid primer design pipeline.

2.1 Background.

Single Nucleotide Polymorphism (SNP) are variations that occur in specific positions of the genome in at least 1% of the population of certain species (Jehan and Lakhanpaul, 2006). In modern breeding programs SNP markers are a prevalent technology to select individual plants or seeds containing a particular locus, which is linked to a trait (ie. a marker linked to a resistance gene, see Chapter 3). SNPs marker is a specific case of Polymerase Chain Reaction (PCR) amplification where two competing sequences from different alleles are amplified. However, in polyploid species the variations between homoeologues are not considered SNPs. Because of the similarity homoeologous regions can interfere in PCR amplification it is preferable to design primers that will take in account the variations between the genomes and the target SNP simultaneously. This chapters describes PolyMarker, a bioinformatic tool to design genome-specific primers.

2.1.1 Primers and Polymerase Chain Reaction

To generate new copies of DNA, biological systems require a DNA polymerase to copy DNA into DNA or a reverse transcriptase to translate RNA to DNA. During DNA replication, the DNA polymerase binds to the template strand and to a short strand of RNA that serves as a primer for DNA synthesis and replication, and then adds complementary nuc-

leotides. Eventually, this results in two complementary DNA strands (Mullis et al., 1987).

This process can be used to generate multiple copies of a DNA sequence using the PCR. PCR uses a heat-stable DNA polymerase to amplify the template sequence. The DNA polymerase requires a primer sequence that is complementary to the template strand to begin the reaction. Usually, chemically synthesised oligonucleotides of approximately 20 bases are added as primers (see Section 2.1.2 for details on primer design). This primer sequence can be specific to the target region, or random if multiple regions are being duplicated. Once the DNA strand has been elongated, the temperature is elevated and then lowered to separate the DNA strands. The process is repeated multiple times to increase the number of DNA sequences exponentially Alberts et al. (2014).

PCR amplification is a technique that can be used to copy a fragment of DNA several times. To start the amplification a pair of sequences (left and right primers) on each side of the target sequence is required. The sequence between primers is copied thanks to the DNA polymerase, an enzyme that moves along the DNA strand making a copy (product). The process starts with an increase in temperature in which the double stranded DNA when the DNA molecule is melted in individual strands. Then, the temperature is dropped so the primers anneal to the DNA strands. Depending on the reaction type the temperature is raised again to 72°C which is the temperature at which Taq polymerase extends. At this point, the polymerase starts extending the strand from the 3'-end of the primer. The temperature is raised again to separate the new strand from the original DNA and lowered again to get the right primer to anneal to the new product. Then, in the extension step, the amplification occurs until the end of template sequence, were the 5'-end of the left primer was originally located. This process is repeated several times to increase the representation of the target DNA (Figure 2.1).

2.1.2 Primer design with Primer3

To initialize de PCR reaction (Section 2.1.1)) it is crucial to design pairs of short DNA sequences, primers, that will start the amplification process. For a primer to be effective it has to be design under certain constrains. Primer3 (Untergasser et al., 2012) is a bioinfrmatic tool to desig primers, considering the following critera:

Figure 2.1: PCR Diagram. PCR is used to amplify a region of the DNA (green bar) target to amplify (product; red line) is found by a pair of primers (blue lines). The 3' and 5' represent the orientation of the primers.

Oligonucleotide melting temperature . It is the temperature at which the double stranded primer melts into single strands. If the melting temperature is too high, the primer will not bind to the target sequence (Breslauer et al., 1986).

Size. The size of the primers needs to be balanced to be long enough to provide the desired specificity to the target seqnece, and short enough to to bind easily to the target sequence. A common range of sizes for priemrs is between 18-25bp.

GC content. Primers with a high GC content are more efficent, however they also tend to be less specific (Rychlik, 1995).

Avoid primer-dimers. In a primer pair, if the corresponding 3' ends are similar the primer pairs may bind to each other. Hence, when selecting a primer pair, even if the individual primers are good candidates, the corresponding pair must be checked to avoid this issue. (Chou et al., 1992).

PCR product size. It is the distance between the 5' end of both primers. The length of the product will determine how many cycles of extension are needed. For SNP markers, a short product size is prefered because the snp is already contained in the primer.

Positional constrains in the template sequence. It is possible to exclude or force the inclusion of certain regions in the primer design. In particular, this capability enables PolyMarker to select primers with the SNP in the 3' end of the first primer for KASP (see Section 2.1.3) and genome-specifc priemrs on the second primer (see Section 2.1.4).

This list is of criteria considered by Primer3 is not comprehensive, but it includes the relevant options considered for primer design in PolyMarker.

2.1.3 KASP assays.

A technology used for genotyping SNP markers is Kompetitive Allele Specific PCR (KASP) assay, the original target technology for PolyMarker. The assay consists on triplets of primers, having a primer for each allele and a common primer that will amplify both alleles. The allelic primers have at the 5'-end a tail, HEX (5' GAAGGTCGGAGTCAACGGATT 3') or FAM (5' GAAGGTGACCAAGTTCATGCT 3'), which is used to distinguish between them (Figure 2.2a). The KASP mix contains complementing oligos to the HEX and FAM tail, which contain a dye that is only visible when the corresponding allele has amplified. The intensity of each dye is used to measure relative amplification of each allele. In KASP assays, the distance between the left and right primers is kept as short as possible, to avoid having an extension step. As the primers are around 21-25bp, the minimum product size is between 42-50bp, with products rarely going over 75bp. Samples with the same genotype cluster: Samples on each axis correspond to homozygous individuals and samples clustered between the homozygous clusters are heterozygous (Figure 2.2b). If the experiment fails, because poor amplification, that means that there are no distinguishable clusters amongst the samples (Figure 2.2c; LGC Genomics 2014).

2.1.4 Genome specific primers.

One of the main challenges of working with polyploid species is the design of genome specific molecular markers. In hexaploid wheat, most of the genes have at three homoeologues copies, one for each genome (See section 1.1.1). The similarity between homoeologues is around 98% (Krasileva et al., 2013), which represent around 1 mismatch for every 50 bp. This means that a primer in a conserved region of 21 bases targets any of the homoeologues if it does not have variations on it. In Figure 2.3, variations between genomes are represented with red lines, which are randomly distributed across homoeologues. The α is randomly generated using the sequence of chromosome 1D, however, because it doesn't have any variation specific to the D genome, products from it can amplify any of the genome. On the contrary, the β starts with a base that is unique to the D genome, hence the product is genome specific.

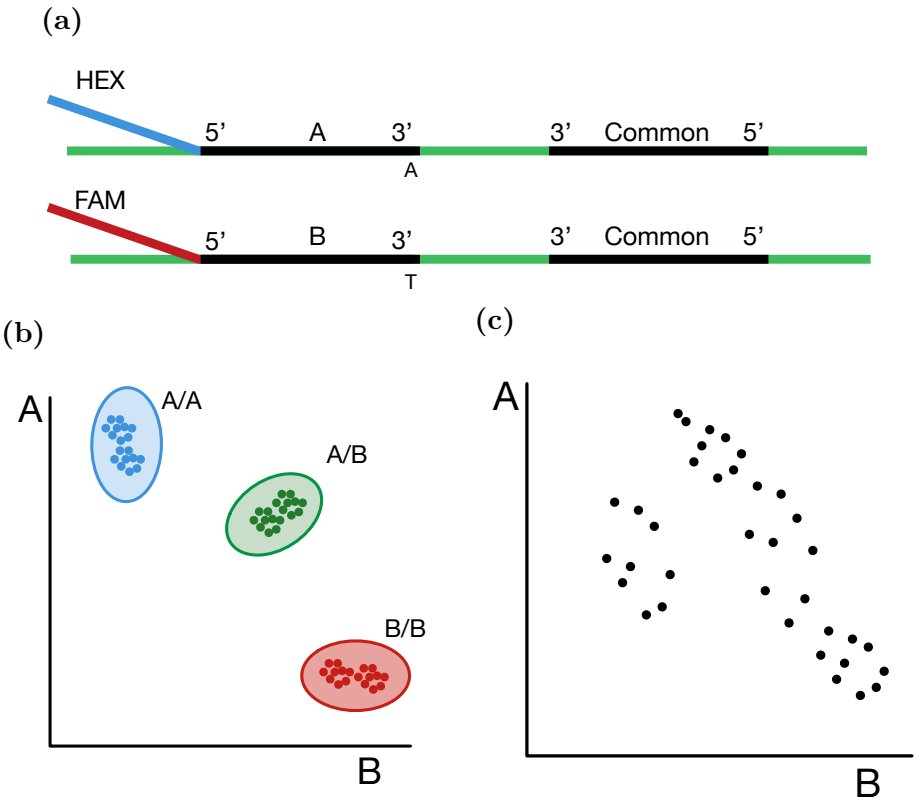


Figure 2.2: KASP Assays (a) A KASP assay consists on three primers. Primers A and B are specific for certain allele and the HEX and FAM tails are added at the 5'-end on each primer. The common primer amplifies both possible products. The SNP is an A/T, the only difference between alleles. (b) Ideal KASP results are obtained when tight and distinct clusters are obtained. The samples containing A allele clusters on the top-left (blue), the B allele cluster on the bottom-right (red) and the heterozygous cluster between the homozygous clusters (green). Each dot represent a sample and the axes are the relative intensity of amplification of each allele. (c) KASP results in an experiment with inconsistent amplification between the two alleles, clear clusters between samples are missing.

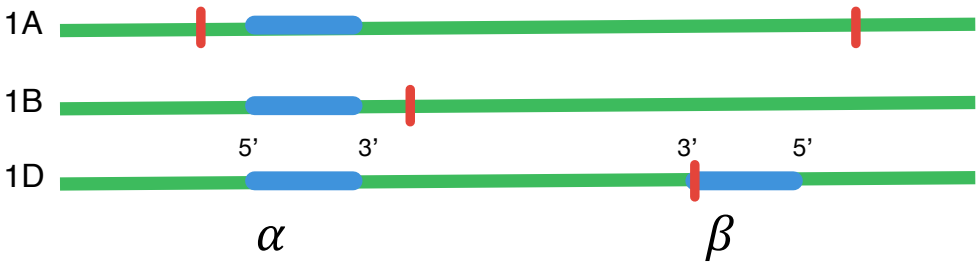


Figure 2.3: Target of genome specific primer. Primers selected randomly (blue lines) can bind to any of the three homoeologous regions if they fall on regions without variations between them (red vertical lines). The α primer doesn't contain any variation between chromosomes, hence it will bind to the chromosomes 1A, 1B and 1D. The β primer has a variation specific to the D genome, hence it will only amplify the 1D chromosome.

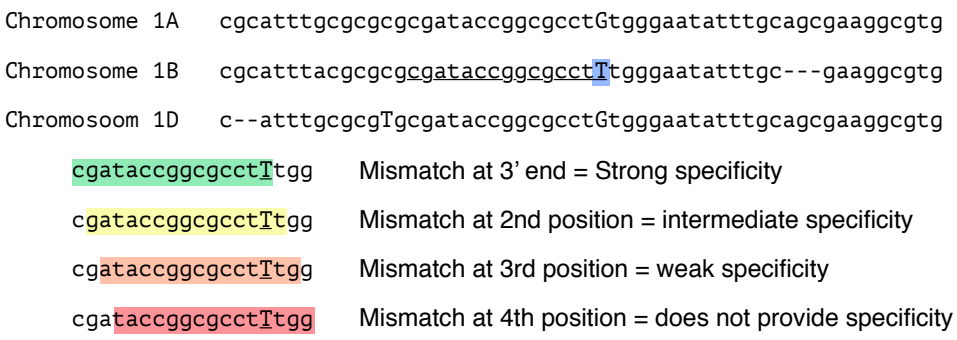


Figure 2.4: Effect of position of variation on primer specificity. Several candidate primers to design a genome specific assay for chromosome 1B are shown. The T highlighted in blue is a variation unique to the target chromosome. The closer the T providing specificity is to the 3' of the primer, the more specific it is.

A variation between homoeologues in the primers is not enough to guarantee that the amplification is going to be genome specific. The polymerase is more specific to variations were the amplification starts, so variations in the 3'-end improve the specificity of primers (Huang and Brûlé-Babel, 2010). Hence, when designing genome-specific assays the specificity of the primers is scored according to the position of the variation as: strong, when the variation is on the 3'-end; intermediate, when the variation is on the 2nd position; weak when the variation is on the 3rd position; and not specific when the variation occurs after the 3rd position (Figure 2.4).

To ensure that all the constrains needed to produce genome-specific primer pairs, the following steps need to be done:

1. First, a global alignment of the target sequence is used to find all the homoeologues and paralogues in the reference genome. This is done with tools like `blast` (Altschul et al., 1990), `blat` (Kent, 2002) or `exonerate` (Slater and Birney, 2005). All of these tools take a reference sequence and make an index of the database to speed up the search of the queried sequence 2.5. Since some of the sources of SNPs come from transcriptome data and gene references, the original sequence may go over the intron-exon junction (see Section 1.2.2). The results are aligned to the target and may include sequences only from one exon, but not the adjacent intron, hence it is necessary to make a local alignment to ensure that corresponding bases are aligned correctly.

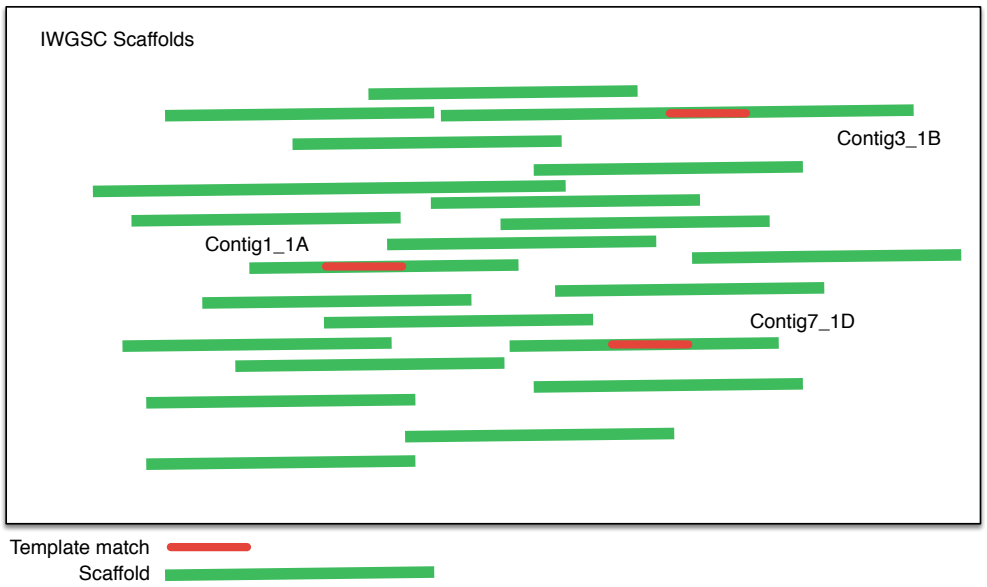


Figure 2.5: Global search of templates in the reference contigs.

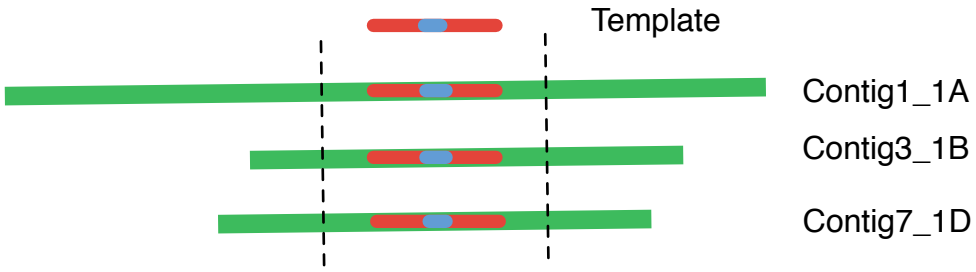


Figure 2.6: Selected regions around the SNP on every chromosome. The blue line represents the position of the SNP.

2. To put all the sequences in the appropriate context, a local alignment is done (Figure 2.6). This is done by extracting all the hits (matches) to the target reference and using a program like `mafft` (Katoh and Standley, 2013) or `clustal` (Higgins and Sharp, 1988). These tools are based on aligning all the possible sequences in pairwise combinations. The distance between pairs is calculated to find which sequences are closer to each other, and then the process is repeated to refine the alignments until a consensus alignment is reached. This is useful on the context of genome-specific primer design because to correct the alignment on the presence of small insertions and deletions (indels).

3. Finally, the primers are validated to conform physicochemical properties that ensure the amplification. The melting temperature needs to be in the range were the DNA will separate, but not

too high that the temperature will damage other elements in the reaction, such as the polymerase. Also, the primers must avoid sequences that self-bind, hairpins, or binding to the complementary primer. The validation primers based on their intrinsic properties can be done with tools like `Primer3` (Rozen and Skaletsky, 2000).

2.1.5 Objective.

Most of the steps required to design genome-specific primers require different bioinformatic tools and the rules to improve the efficiency of the primers are established. The objective of PolyMarker is to automate the primer design process, from a reference genome and a list of SNPs and produces genome-specific primers. The pipeline has been published in Ramirez-Gonzalez et al. (2015a).

2.2 Pipeline.

PolyMarker is an automated pipeline that takes as input a list of SNPs and a reference file and produces a list of primer triplets for SNP genotyping. The list of SNPs is first converted to a `FASTA` file with ambiguity codes (Cornish-Bowden, 1985) The template sequences are aligned with `exonerate` (Slater and Birney, 2005) to find the homoeologous and paralogue regions to the target sequence. For my thesis, I implemented this using the IWGSC reference sequence (described in Chapter 1.4). Then, the alignment between homoeologues is refined using `MAFFT` (Katoh and Standley, 2013). A list of candidate variations is produced and used as input for `Primer3` (Rozen and Skaletsky, 2000). Finally, the output of `Primer3` is parsed to select the shortest primer pair containing the targeted SNP and a base that is specific to the target genome (Figure 2.7). The pipeline is written as a Ruby script, using parsers and wrappers from BioRuby (Goto et al., 2010) and bio-samtools (Etherington et al., 2015; Ramirez-Gonzalez et al., 2012). The software is open source and released as a biogem (Bonnal et al., 2012), `bio-polyploid-tools`, the source code is available in: `https://github.com/TGAC/bioruby-polyploid-tools`.

The PolyMarker input consists on SNP list with: unique name for the marker, the target chromosome and the sequence for the marker. The alternative alleles are flanked by square brackets within the sequence.

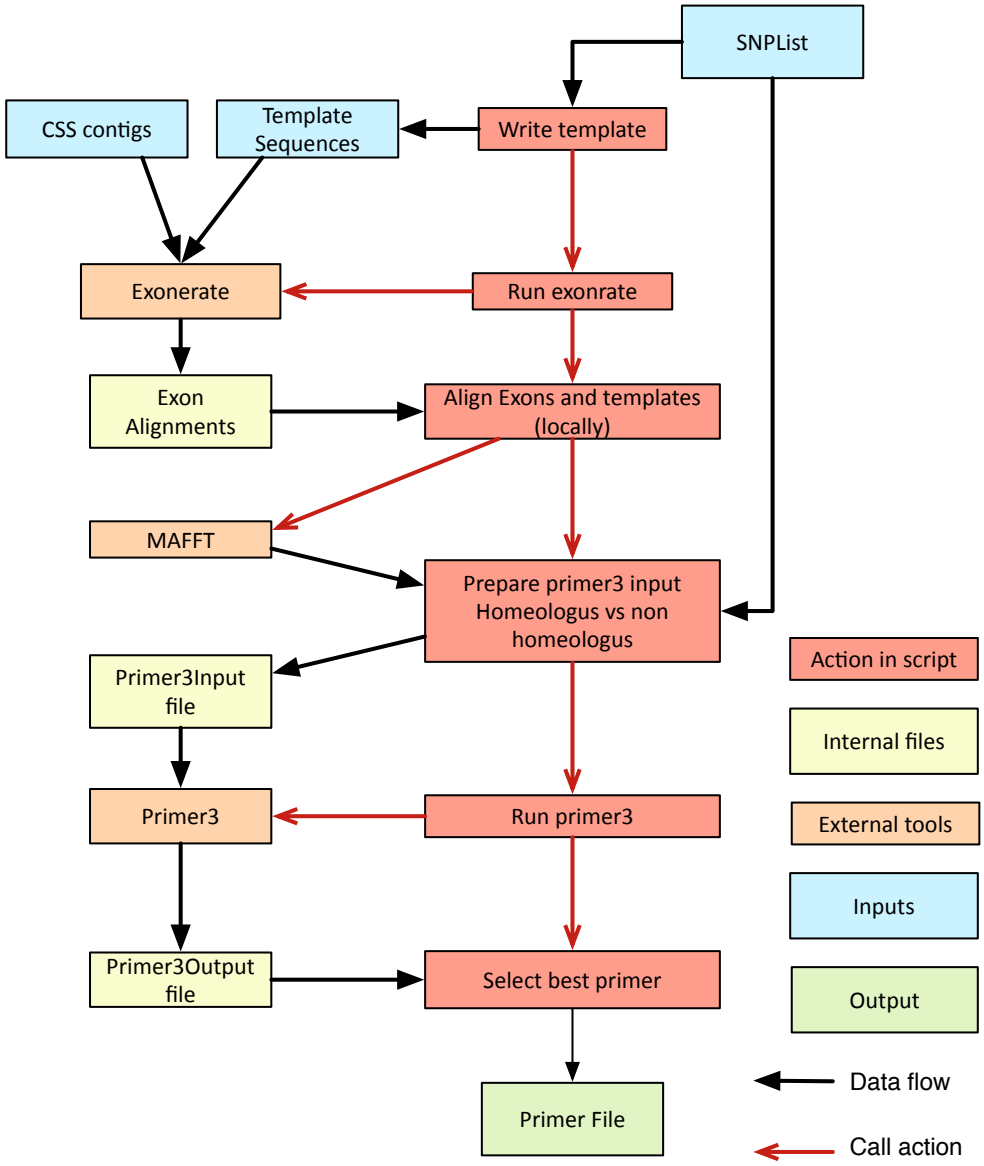


Figure 2.7: Steps and tools called by PolyMarker. The colours of the boxes represent: the step is an action inside the script(red); actions of the script(light red); temporary files(yellow); inputs(blue) and; outputs(green)

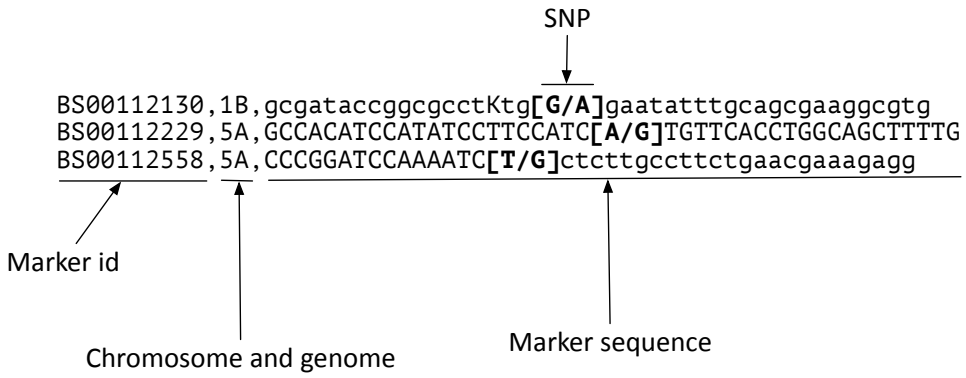


Figure 2.8: PolyMarker input. The alternative alleles are surrounded by brackets. The rest of the figures are based on BS00112130, renamed as SNP-1.

PolyMarker can take a list of several markers and design them in batch (Figure 2.8). A `FASTA` file is produced with all the template sequences, with the alternative alleles substituted by the IUAPC ambiguity codes (Cornish-Bowden, 1985). The flanking sequence surrounding the SNP is limited by default to 100bp to reduce the search time and avoid missing regions that diverge near the SNP, as when the variation is near an intron-exon junction. The limitation of the flanking sequence to +/- 100 is consistent with the marker assay which is restricted to amplicons of 100-120 bp.

The template sequences are aligned to the reference using `exonerate` (Slater and Birney 2005; Figure 2.5). The following parameters are used to optimise the output:

`-verbose 0 -show -alignment no -show vulgar no.` To override the default output.

`-bestn 20.` By default, it increases the number of best hits to 20. Intuitively, it would be expected to have 3 copies, on for each homoeologue. However, the CSS assembly has some duplication in the scaffolds and it is possible to find paralogues elsewhere in the genome.

`-model est2genome.` To allow the search of sequences coming from transcripts, such as the SNPs described in Chapter 3 and in the SNP chip described by (Allen et al., 2011)

`-ryo 'RESULT:\t%S\t%pi\t%ql\t%tl\t%g\t%\n'.` To set the output in a tabular format that is easy to parse as follow: `\%S` the minimum

```
SNP-1 A     cgcatttGcgcgYgcgataccggcgcctKtgGgaatatttgcagcgaaggcgtg
SNP-1 B     cgcatttAcgcgYgcgataccggcgcctKtgAgaatatttgcagcgaaggcgtg
IWGSC-1A    cgcatttgcgcgcgcgataccggcgcctgtgggaatatttgcagcgaaggcgtg
IWGSC-1B    cgcatttacgcgcgcgataccggcgcctttgggaatatttgcgaaggcgtg
IWGSC-1D    catttgcgcgTgcgataccggcgcctgtgggaatatttgcagcgaaggcgtg
```

Figure 2.9: Sequence of flanking regions around the SNP. The indels produce a slight shift on the sequence.

information of the alignment, `\%pi` percentage of identity, `\%ql` query length, `\%tl` target length and, `\%g` orientation.

All the hits that contain the SNP and have a percentage of identity over 90% are extracted, this threshold allows to match homoeologs and paralogs. The coordinate of the SNP is calculated and 100bp on each flank are extracted by default, a reasonable product size for KASP assays. The flanking sequence may contain indels and the sequences do not align naturally (Figure 2.9). The following parameters can be adjusted to extend the functionality of PolyMarker: Minimum Identity to designs for organisms with homoeologous regions that are more divergent; flanking sequence for different types of primers (ie. for Sanger sequencing) and; `model` to adjust the search according to the source of the SNP (ie. if it is known that the SNP comes from DNA, `affine:local` would be a better option as `exonerate` will not pay attention to the intron-exon junctions).

Each SNP marker is represented on the `Bio::PolyploidTools::SNP` class, containing the flanking sequence, the position of the SNP, multiple alignments and primers. For each step step there is a container that holds the SNP set and parses each output for all the called programs. The container for exonerate is `BIO:PolyploidTools::ExonContainer`. The hits with the SNP are called exon henceforth, as the original design was for SNPs in gene models which may contain intron-exon junctions. The main job of the `ExonContainer` is to parse the `exonerate` output and add it to the corresponding SNP (Listing 2.1).

Listing 2.1: Method in `Bio::PolyploidTools::ExonContainer` that adds to each SNP object the alignments

```
def add_alignments(opts=Hash.new)
  opts = {:min_identity=>90 }.merge!(opts)
  exonerate_filename = opts[:exonerate_file]
  File.open(exonerate_filename) do |f|
    f.each_line do | line |
      record=Bio::DB::Exonerate::Alignment.parse_custom(
          line)
      if record and record.identity>=opts[:min_identity]
        snp_array = @snp_map[record.query_id]
        snp_array.each do |snp|
        if snp.position.between?( (record.query_start +
            1) , record.query_end)
          exon=record.exon_on_gene_position(snp.position
              )
          snp.add_exon(exon, arm_selection.call(record.
              target_id))
        end
      end
    end
  end
end
```

Each `SNP` contains a Hash to the best alignment to each chromosome, based on identity. When the `ExonContainer` adds an alignment, the `SNP` verifies that is the best hit for a given chromosome, to avoid scaffolds with duplicated sequences (Listing 2.2).

Listing 2.2: Method in `Bio::PolyploidTools::SNP` that adds an alignment

```
def add_exon(exon, arm)
  @exon_list[arm] = exon unless @exon_list[arm]
 @exon_list[arm] = exon if exon.record.score >
     @exon_list[arm].record.score
end
```

As it is common to have different conventions over different references on how the chromosomes are named, PolyMarker can be easily extended to parse different naming conventions. To achieve this, when the `ExonContainer` is initialised a parsing function is set up. Then, when

```
SNP-1 A     cgcatttGcgcgYgcgataccggcgcctKtgGgaatatttgcagcgaaggcgtg
SNP-1 B     cgcatttAcgcgYgcgataccggcgcctKtgAgaatatttgcagcgaaggcgtg
IWGSC-1A    cgcatttGcgcgcgcgataccggcgcctGtgGgaatatttgcagcgaaggcgtg
IWGSC-1B    cgcatttAcgcgcgcgataccggcgcctTtgGgaatatttgc---gaaggcgtg
IWGSC-1D    c--atttGcgcgTgcgataccggcgcctGtgGgaatatttgcagcgaaggcgtg
```

Figure 2.10: Local alignment on regions around the SNP detects indels.

each alignment is added, the ID of the target sequence is parsed using the custom function (Listing 2.1, line 12). An example of parsing functions for a chromosome are in Listing 2.3.

Listing 2.3: Example function that assigns a chromosome from the two first letters of the scaffold

```
1 arm_selection_functions[:arm_selection_first_two] =
      lambda do | contig_name |
2   ret = contig_name[0,2]
3   return ret
4 end
```

To ensure that the indels between homoeologues do not produce spurious mismatches a local alignment is produced with `MAFFT` (Figure 2.10). The arguments used are the recommended ones in the manual for small number of sequences:

`-maxiterate 1000`. The local alignment is defined up to 1000 times.

`-localpair`. Compares all the possible pairs of alignment to each other

`-quiet`. To reduce the size of the logs.

The class `Bio::PolyploidTools::SNP` has the method `aligned_sequences` which executes `MAFFT` for the best hit on each chromosome to the marker. The first time it is invoked it stores the result as an attribute (Listing 2.4). This approach hides the execution of the local alignment as an attribute and it avoids executing it several times when calculating the variations between homoeologues.

Listing 2.4: Method in `Bio::PolyploidTools::SNP` that calculates the local alignment

```
1 def aligned_sequences
2   return @aligned_sequences if @aligned_sequences
3   options = ['--maxiterate', '1000', '--localpair', '--
       quiet']
4   mafft = Bio::MAFFT.new( 'mafft' , options)
5   report = mafft.query_align(sequences_to_align)
6   @aligned_sequences = report.alignment
7   @aligned_sequences
8 end
```

PolyMarker searches across each base in the local alignment to identify the variations across homoeologues and the target marker. A mask is produced to highlight the bases with a variations, Figure 2.11, on the following categories:

Specific — Homoeologous polymorphism which is only present in the target genome (upper case).

Semi-specific — Homoeologous polymorphism is found in 2 of the 3 genomes, hence it discriminates against one of the off-target genomes or when not all the homoeologous sequences were found (lower case).

Non-specific — No variation is found across homoeologues (-).

Homoeologous — The target SNP is present across different chromosomes, so candidate SNP markers on this category are not expected to be reliably identifying the allele as these are not necessarily varietal polymorphisms (:).

Non-homoeologous — The target SNP is not present across chromosomes, so it is most likely a varietal polymorphism which can be used to identify alternative alleles in the position (&).

To generate the mask the following logic is followed:

1. The aligned sequence of the target chromosome is set up as the default mask (Listing 2.11, line 5).

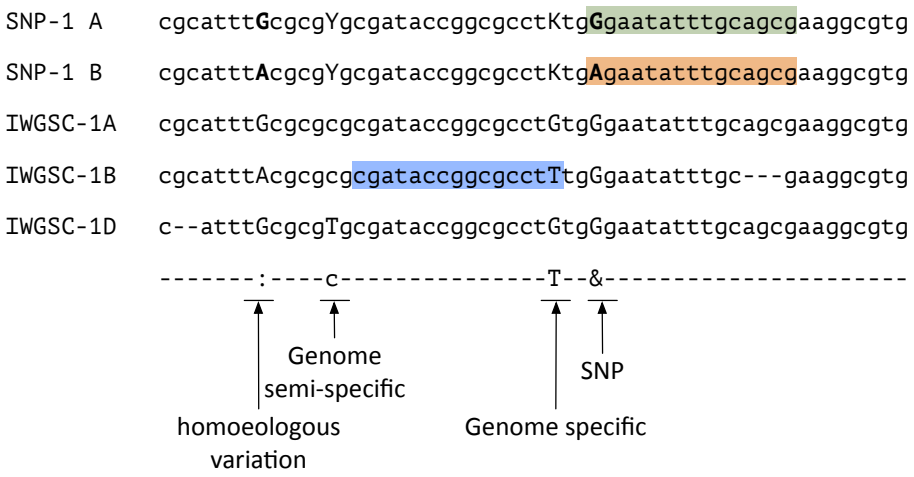


Figure 2.11: Alignment with mask and primer candidates. The green and light red boxes highlight the allele specific primers. The blue box highlights a genome specific primer.

2. Then each position in the mask is iterated base per base (Listing 2.11, line 7).

3. A count of how many bases are the same across the chromosome and how many from the same chromosome group (defined by the first letter in the parsed chromosome) and how many chromosomes have local alignment (excluding indels; lines 9-18).

4. A position is labelled as uninformative (`-`) when the position does not have any different bases, the sequence is only available from the target chromosome or there are unknown bases on that particular position (any chromosome has an `N` on the given position; line 19).

5. When no alignment is present at all, the mask is filled with `*` (line 20). This allows to identify the cases where only the initial marker sequence is available.

6. If the target chromosome has an unique variation, the base is converted to upper case (line 22). This implicitly leaves as a lower case the semi-specific variations. The `genomes_count` is a variable set at initialisation time and keeps track of the number of expected alignments from the target group. This allows to use the same code for any level of polyploidy.

7. At the position of the SNP, the special symbols are setup (lines 23-30)

(a) By default, the SNP position is labelled as `&` (line24).

(b) All the observed bases, except the one in the target chromosome, are collected and converted to an IAUP ambiguity code (Cornish-Bowden, 1985) (lines 26-28). If the bases in the SNP are contained in the ambiguity code the SNP is marked as homoeologous (`:`; line 29)

When designing SNP markers the aim is to have the amplification as specific as possible. To improve the specificity of the assays, PolyMarker categorises all the possible primers as Specific; Semi-specific or; Non-specific. The candidate primer pairs are then evaluated with `Primer3` (Rozen and Skaletsky, 2000). `Primer3` receives a file with the preferences to design the markers, for PolyMarker the following preferences are set up:

`PRIMER_PRODUCT_SIZE_RANGE=50-150.` A reasonable size for KASP markers, as the technology does not have an extension step.

`PRIMER_MAX_SIZE=25.` KASP primers are usually between 21 and 25 bases.

`PRIMER_LIB_AMBIGUITY_CODES_CONSENSUS=1.` To ensure that bases with ambiguity code are matched between primer pairs.

`PRIMER_LIBERAL_BASE=1.` To allow the use of ambiguity codes in the sequence

`PRIMER_NUM_RETURN=5.` The maximum number of primer candidates.

To design a different kind of primers it is possible to have a different set of preferences by feeding a standard `Primer3` preferences file with the option `--primer_3_preferences FILE`.

The input file for `primer3` also include the template sequences with an ID. To keep track of what kind of marker each position will produce the ID field has the name of the primer and the specificity of the starting position of the common primer. The starting position of the primers is forced with the options `SEQUENCE_FORCE_LEFT_END` and `SEQUENCE_FORCE_RIGHT_END` on the specific and semi-specific positions. For the non specific positions only the `SEQUENCE_FORCE_LEFT_END` is given to make a full search of candidates.

Listing 2.5: Method in `Bio::PolyploidTools::SNP` that calculates the mask of the alignment

```
def mask_aligned_chromosomal_snp(chromosome)
  names = exon_sequences.keys
  parentals =  parental_sequences.keys
  local_pos_in_gene = aligned_snp_position
  masked_snps = aligned_sequences[chromosome].downcase
  i = 0
  while i < masked_snps.size
    different = cov = from_group = Count = 0
    names.each do | chr |
      if aligned_sequences[chr] and aligned_sequences[
          chr][i]  != '-'
        cov += 1
        nCount += 1 if aligned_sequences[chr][i] == 'N'
           or  aligned_sequences[chr][i] == 'n'
        from_group += 1 if chr[0] == chromosome_group
        if chr != chromosome
          different += 1  if masked_snps[i].upcase !=
              aligned_sequences[chr][i].upcase
        end
      end
    end
    masked_snps[i] = '-' if different == 0 or if cov ==
       1 or nCount > 0
    masked_snps[i] = '*' if cov == 0
    expected_snps = names.size - 1
    masked_snps[i] = masked_snps[i].upcase if different
       == expected_snps and from_group == genomes_count
    if i == local_pos_in_gene
      masked_snps[i] = '&'
      bases = ''
      names.each do | chr | { bases << aligned_sequences
          [chr][i]  if aligned_sequences[chr] and
          aligned_sequences[chr][i]  != '-' }
      code_reference = 'n'
      code_reference = Bio::NucleicAcid.to_IUAPC(bases)
          unless bases == ''
      masked_snps[i] = ':' if Bio::NucleicAcid.is_valid(
          code_reference,   original) and Bio::
          NucleicAcid.is_valid(code_reference,   snp)
    end
  i += 1
  end
  masked_snps
end
```

The class `Bio::DB::Primer3::Primer3Record` is used to keep the details of all the primers generated by `primer3` for each template. In order to prioritise which primer is selected as the best primer on for each SNP, each `Primer3Record` is scored according to their type and the product length (Listing 2.6). By default, more priority is given to the specific, semi-specific and non-specific primers, in that order. In case of having more than one primer pair with the same specificity, the one with the shortest product length is chosen (Listing 2.7).

Listing 2.6: Method that calculates the score of a primer `Bio::DB::Primer3::Primer3Record`

```
1 def score
2   ret = 0
3   ret += @scores[type]
4   ret -= product_length
5   ret
6 end
```

Listing 2.7: Initialisation of the `Bio::DB::Primer3::Primer3Record` class, including the default score weights

```
1 def initialise
2   @properties = Hash.new
3   @scores = Hash.new
4   @scores[:chromosome_specific] = 1000
5   @scores[:chromosome_semispecific] = 100
6   @scores[:chromosome_nonspecific] = 0
7 end
```

Finally, the best primer for each marker is produced and a CSV file is produced with the following columns:

Marker The ID of the Marker

SNP The position of the SNP in the original sequence and the kind of SNP

RegionSize The size of the original sequence tested, up to the maximum size including the flanking sequence.

chromosome The target chromosome

total_contigs How many contigs mapped to the SNP. If it is more than the expected by the ploidy of the organism it can show paralogues or repetitive regions.

contig_regions The locations where the marker mapped. In the format Scaffold:start-end

SNP_type homoeologous or non-homoeologous. If it is homoeologous, the SNP is probably a variation between chromosomes.

A Primer for the first allele.

B Primer for the second allele.

common Common primer that gives the specificity to the assay.

primer_type specific, semi-specific or non-specific. Depending on the rules described previously.

orientation If it is forward, the allelic primers are in the same orientation as the original sequence. If it is reverse, the common primer is in the same orientation as the original sequence.

A_TM Melting temperature of the first allelic primer

B_TM Melting temperature of the second allelic primer

common_TM Melting temperature of the common primer

selected_from For internal purposes, points from which of the primers was used as template.

product_size The size of the PCR product produced by the primers.

PolyMarker also produces a text file with the local alignments that contain all the positions that can produce a genome-specific primer. The file has the same format as Figure 2.11, but without the highlights. The mask is useful in case that the original assay failed, or to explore the details of the other homoeologs and paralogs which are similar to the assay.

2.2.1 PolyMarker public web service

To make PolyMarker accessible to the community, a web server that allow the submission of SNPs was developed. The web interface consists on two virtual machines, one with a web-facing interface that stores the queries, and a dedicated node to submit jobs to an HPC cluster. The online interface further simplifies the design of KASP assays, a process that used to take between 15-45 minutes per marker is now automated. Since the release of the public service in July 2014 until August 2016, 1,739 requests to PolyMarker have been done, demonstrating the popularity of the PolyMarker tool.

Besides the previously described output, the web interface of PolyMarker provides a graphical representation of the multiple sequence alignment and the mask used to design the primer (Figure 2.12). The visualisation consists on a table containing the primers and the BioJS component MSAViewer (Yachdav et al., 2016), that highlights the designed primers. On an ideal case, you have an SNP that is in a non-homoeologous position with a genome-specific triplet (Figure 2.12a). However, sometimes the SNP is located in an homoeologous variation (Figure 2.12b), which can signal a miscalled SNP. In some extreme cases, a SNP is located in regions that have homoeologues and paralogs in several chromosomes (Figure 2.12c), it is useful to highlight such kind of SNPs that can produce spurious amplification from non-target chromosomes. The graphical representation is helpful to understand how the primers were designed.

2.3 Applications of PolyMarker

Besides the project described in Chapter 3, PolyMarker has been used to design KASP primers for the wheat community.

2.3.1 KASP assays for public sets of SNPs

PolyMarker was used to design KASP assays for the 81,587 markers from (Wang et al., 2014), available on the PolyMarker website and in CerealsDB (Wilkinson et al., 2012). Of those markers, 40,267 where designed based on the target chromosome from the genetic map provided in Wang et al. (2014). Genes without a genetic position were aligned

Figure 2.12: Examples outputs from the PolyMarker website. (a) The primer triplet is genome specific. The original marker sequence had the SNP near the beginning of the template, but PolyMarker used the genomic reference to complement the sequence. (b) An specific primer, but the SNP is located on the same position than an homologous variation. (c) A case where the marker sequence align to 10 different chromosomes. The SNP is also located on a position with variations between genomes.

Table 2.1: Count of KASP assays designed for the 40,267 SNP markers located in the genetic map from Wang et al. (2014). 4,228 assays did not align to the target chromosome. Not designed: Primer3 could not find viable primers flanking the SNP.

	Homoeologous variant	Varietal SNP	Percentage
Non-specific	1,765	5,857	21.15%
Semi-specific	7,942	6,907	41.20%
Specific	6,813	5,957	35.43%
Not designed	242	556	2.21%
Total	16,762	19,277	36,039

to scaffolds sorted by chromosome arms from the International Wheat Genome Sequencing Consortium (Mayer et al., 2014) with BLAT (Kent, 2002) and the best hit was selected as the putative location. 97.5% of the assays where designed and 76% of them are semi-specific or specific, thereby improving their expected performance with respect to randomly designed primers (Table 2.1). The pre-designed markers have been taken up by the community, for example, a subset of the designed assays was used to genotype a mapping population to find resistance to Fusarium head blight (Burt et al., 2015).

Also, PolyMarker was used to design KASP assays for the 820K SNP Axiom array described in Winfield et al. (2016). Briefly, the original set contains 819,556 SNPs called from exome capture on 43 bread wheat accessions and wheat relatives. Of those, 616,525 where mapped with `exonerate` (Slater and Birney, 2005) to the CSS scaffolds. Of those, 86.1% have an specific or semi-specific assay (Table 2.2. This set of primers is also available in CerealsDB and it provides a valuable resource to groups that want to genotype using a subset of SNPs in the array, without the need to run the complete Axiom array. This is especially relevant in breeding programmes who might want to run a small subset of markers linked to their favourite traits. The fact that the assays could be downloaded all in one makes it difficult to document the impact, but based on conversation with molecular breeders we are aware that they are being implemented in several breeding companies.

Table 2.2: Count of KASP assays designed for the 616,525 SNP markers located to a CSS scaffold from the 819,556 SNPs from Winfield et al. (2016) Not designed: Primer3 could not find viable primers flanking the SNP.

	Homoeologous variant	Varietal SNP	Percentage
Non-specific	20,189	56,516	12.44%
Semi-specifc	167,018	132,145	48.52%
Specific	139,202	92,487	37.58%
Not designed	3,116	5,852	1.45%
Total	329,525	287,000	616,525

2.3.2 SNPs in a mutant population

PolyMarker was used to design primers to validate SNPs in a Targeted Induced Local Lesions in Genomes (TILLING) population, an approach to identify the function of genes by mutating them. Briefly, wheat lines are mutated with ethyl methanesulphonate that produce G>A or C>T mutations. The initial mutation is called M_1 and each plant is self-pollinated to fix the mutations. The second generation is called M_2, and so on. With each generation the mutations, which were originally heterozygous, get fixed and become homozygous. In the process, some mutations are lost. For this experiment, three M_5 lines were sequenced with exome capture. The purpose of the experiment was to assess the feasibility of exome capture for call for SNPs.

To validate the SNPs detected at different levels of coverage and allele frequencies 150 assays were designed. The assays were tested on the M_5 used for SNP calling and on the progenitors at M'', M_3. Most of the SNP calls with more than 8 variant calls or an allele frequency over 0.8 were validated (Table 2.3). At the same time, only 27% of the SNPs with an allele frequency of 0.6 and 17% of the cases with seven or less variant reads were successful. (King et al., 2015). On this experiment PolyMarker was useful on validating and calibrating the minimum coverage to call SNPs reliably.

On a follow-up experiment consisting of 1,200 Cadenza (Hexaploid) and 1,535 Kronos (Tetraploid) wheat lines (Krasileva et al., submitted 2016) over 250 SNPs were experimentally validated where also validated. Genome-specific primers were designed for 172 and 80 SNPs in the Cadenza and Kronos populations, respectively. These mutations were

Table 2.3: Summary table of the validation of candidate SNPs by KASP marker assays. Candidate SNPs are classified by number of supporting variant reads or by allele frequency and validated by KASP assays. Table from King et al. (2015).

Criterion	Number/ Frequency	KASP assays	Validated SNPs	Validated (%)
Variant reads	4	25	1	4%
	5	17	3	18%
	6	14	2	14%
	7	14	3	21%
	8	10	5	50%
	9	12	9	75%
	>10	35	29	83%
Allele frequency	0.2	51	2	4%
	0.4	27	13	48%
	0.6	18	9	50%
	0.8	3	2	67%
	1	31	27	87%

spread across 19 and 8 M_4 Cadenza and Kronos lines respectively. Of those, 71(85.5%) Kronos and 147(88.8%) of the Cadenza primers where valid assays, consistent with the pilot study (Tables A.1 and A.2).

2.4 Modifications of PolyMarker

PolyMarker is not restricted to wheat or to KASP assays, the source code is flexible and can be extended for other types of analyses. On each of the following projects, PolyMarker has been adapted to design primers in species where KASP has not been used before, the primers are used for regular PCR amplification, or the use of KASP is not the conventional SNP calling.

2.4.1 Deletions on a mutant population

On some of the TILLING mutant lines, long deletions spanning multiple scaffolds were detected (Krasileva et al., submitted 2016). To validate the deletions it is possible to use KASP assays to produce primers that amplify homoeologues. PolyMarker was modified to search for variations across homoeologues to select a common primer that will amplify two

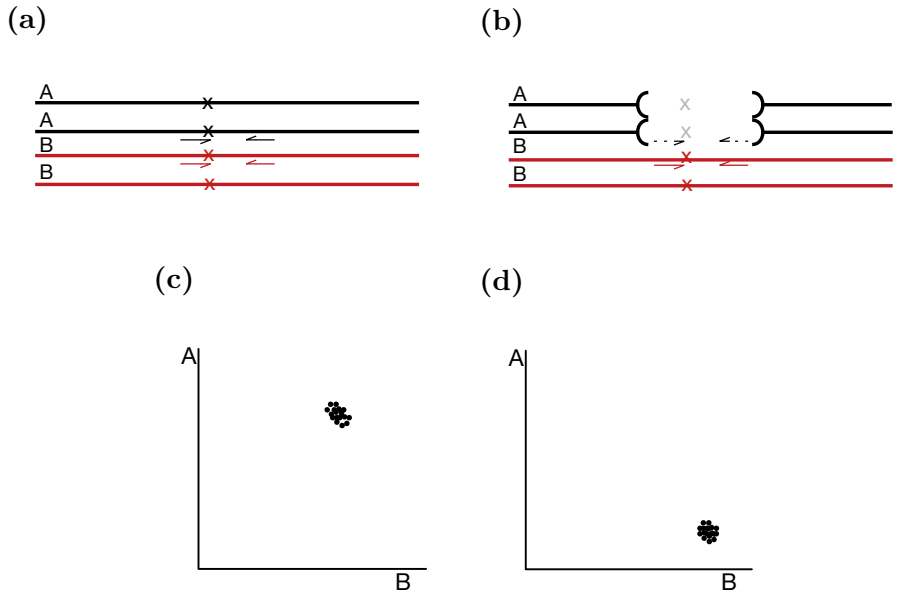


Figure 2.13: KASP assays to validate homozygous deletions. (a) Primer positions for wildtype. Red and black indicate the A and B genome respectively. Primers are indicated by arrows with the target homoeologous SNP marked by an "X"(b) Primer positions on homozygous deletion on M_4 (c) Heterozygous amplification on wildtype DNA (no deletion), including both homoeologues. (d) Homozygous amplification on deletion line, only the non-deleted homoeologue is amplified.

genomes (Figure 2.13a, b; reverse primer). On lines without the targeted deletion, the amplification corresponds to an heterozygous assay with equal signal for both the A and the B allele (Figure 2.13c). When a deletion is present the results of the assay resemble the results for a homozygous individual, with the intensity of the assay towards the the conserved homoeologue (Figure 2.13d).

To be able to select primers that will amplify two homoeologues, the default scoring values (Listing 2.7) are changed. The altered scoring gives priority in the following order semi-specific, non-specific and specific. The rest of the pipeline is unaltered, showing that the modular design allows to add new functionality without breaking the pipeline.

Listing 2.8: Score values to select semi-specifc primers

```
1 kasp_container.scores[:chromosome_specific] = 0
2 kasp_container.scores[:chromosome_semispecific] = 1000
3 kasp_container.scores[:chromosome_nonspecific] = 100
```

A set of KASP assays for the the deletions and mutations located on the same chromosome where designed to validate 11 homozygous deletions on M_4 plants. In all cases the segregation of the mutations was as expected, except for a predicted heterozygous mutation that was called as homozygous. Also, all the KASP assays that contained a deletion were called homozygous, as expected. To ensure that the calls did not come from a single cluster, 4 wildtype plants were genotyped and the markers for deletions where called as heterozygous. An example of a validated deletion and the surrounding mutations, with the calls for each individual is shown on Table 2.4.

2.4.2 Genotyping *Puccinia striiformis* f. sp. *tritici* isolates.

In Hubbard et al. (2015), *Puccinia striiformis* f. sp. *tritici* (PST) isolates were sequenced and assigned to clusters, according to their genotype. The clusters are useful to monitor the changes in the pathogen population, which can be used to predict if certain wheat lines will be resistant to the isolates in the field. PST is a dikaryon, an organism with two nuclei, each one containing a single haploid chromosome. For PolyMarker it can be treated as a diploid, so the `--genomes_count 1` argument was used. PolyMarker was used to design primers for PST, using the assembly PST-130 (Cantu et al., 2011a). As the assembly is fragmented, an *ad hoc* function was used to always get the name of the assembly (Listing 2.9). Out of 15 assays, 11 can be used to identify to which cluster of isolates a sample is likely to belong, Table 2.5. Until this study, the previous method to genotype PST was Simple Sequence Repeat (SSR) markers which were difficult to replicate across laboratories and interpret in cases of multiple alleles (Ali et al., 2014).

Listing 2.9: Function that always returns PST130 as chromosome

```
1 arm_selection_functions[:pst130] = lambda do |
    contig_name|
2   return "PST130"
3 end
```

Table 2.4: Validation of homozygous deletions on line Cadenza0423. X represents a mutant call, Y an wild type call and H an heterozygous call. In the case of the deletions, heterozygous calls are expected in the control lines (C), as two genomes are amplifying.

Marker	Deletion	chr	cM	1	2	3	4	5	6	7	8	9	10	11	12	C	C	C	C	Result
5BS_2297308_Cadenza0423_12664_C12664T	-	5B	4.551	X	X	-	X	X	X	X	X	X	X	-	X	Y	Y	Y	Y	HOM Mutation
5BL_10812849_Cadenza0423_5664_G5664T	-	5B	38.769	X	X	-	X	X	X	X	X	X	X	-	X	Y	Y	Y	Y	HOM Mutation
5BL_10825062_Cadenza0423_7917_G7917A	-	5B	38.769	X	X	-	X	X	X	X	X	X	X	-	X	Y	Y	Y	Y	HOM Mutation
IWGSC_CSS_5BL_scaff_10847976:27068-27231	+	5B	38.769	X	X	-	X	X	X	X	X	X	X	-	X	H	H	H	H	Hom Deletion
IWGSC_CSS_5BL_scaff_10847976:28118-28674	+	5B	38.769	X	X	-	X	X	X	X	X	X	X	-	X	H	H	H	H	Hom Deletion
IWGSC_CSS_5BL_scaff_10865441:15863-15946	+	5B	38.769	X	X	-	X	X	X	X	X	X	X	-	X	H	H	H	H	Hom Deletion
5BL_10837222_Cadenza0423_4616_G4616A	-	5B	39.905	X	X	-	X	X	X	X	X	X	X	-	X	Y	Y	Y	Y	HOM Mutation
5BL_10891320_Cadenza0423_18847_C18847T	-	5B	45.594	Y	Y	-	Y	H	X	X	Y	H	Y	-	H	Y	Y	Y	Y	HET Mutation

Table 2.5: PolyMarker used to genotype PST. The X and Y represent the the two possible alleles. X:X and Y:Y correspond to homozygous call of the corresponding allele. X:Y correspond to heterozygous calls. The '-' symbol correspond to failed assays.

					Cluster I isolates		Cluster II isolates		Cluster III isolates			Cluster IV isolates	
Assay	Contig	Position	X	Y	13/26	13/123	CL1	T-13/3	13/09	13/23	13/182	13/36	13/40
1	PST130_14470	268	C	T	X:Y	X:Y	X:X	X:X	X:X	X:X	X:X	X:X	X:X
2	PST130_8160	11876	C	T	Y:Y	Y:Y	X:Y	X:Y	X:Y	X:Y	X:Y	X:Y	X:Y
3	PST130_14628	1712	A	C	X:Y	-	X:X	X:X	X:X	X:X	X:X	X:X	X:X
4	PST130_14898	503	G	A	X:X	X:X	X:Y	X:Y	X:Y	X:Y	-	X:Y	X:Y
5	PST130_28344	2372	A	G	Y:Y	Y:Y	X:Y	X:Y	Y:Y	Y:Y	Y:Y	Y:Y	Y:Y
6	PST130_7634	3463	A	C	Y:Y	Y:Y	X:Y	X:Y	Y:Y	Y:Y	Y:Y	Y:Y	Y:Y
7	PST130_7629	11699	G	A	Y:Y	Y:Y	X:Y	X:Y	Y:Y	Y:Y	Y:Y	Y:Y	Y:Y
8	PST130_10943	2979	C	T	X:Y	X:Y	X:Y	X:Y	X:X	X:X	X:X	X:Y	X:Y
9	PST130_10126	6216	G	T	Y:Y	Y:Y	X:X	X:X	X:X	X:X	-	Y:Y	Y:Y
10	PST130_22010	172	C	T	Y:Y	Y:Y	Y:Y	Y:Y	X:Y	X:Y	-	X:Y	X:Y
11	PST130_16961	1098	C	T	X:X	X:X	X:Y	X:Y	Y:Y	Y:Y	Y:Y	X:Y	X:Y
12	PST130_6915	2710	A	T	Y:Y	Y:Y	Y:Y	Y:Y	Y:Y	X:Y	X:Y	Y:Y	Y:Y
13	PST130_12479	1428	C	T	X:X	X:X	Y:Y	Y:Y	X:X	X:X	X:X	Y:Y	X:X
14	PST130_7634	3883	C	G	X:X	X:X	X:Y	X:Y	X:X	X:X	X:Y	X:Y	X:X
15	PST130_14470	456	T	C	Y:Y	Y:Y	X:Y	X:Y	Y:Y	Y:Y	X:Y	Y:Y	Y:Y

2.5 Discussion

Before the Chinese Spring Chromosome arm survey sequence (CSS) assembly, which has scaffolds assigned to a chromosome arm, the design and validation of genome-specific primers for polyploid wheat was a labour intensive process (Figure 2.14; Akhunov et al. 2010). Briefly, the steps to develop primers for wheat was:

1. **Find candidate ESTs** from a database. This could be the UniGene database from the National Center for Biotechnology Information (NCBI) or gene sequences from a relative species, such as *Brachypodium distachyon*.

2. **Design primers from UniGenes.** The sequence of the ESTs used as a reference are aligned across them. The conserved regions are used to design primers to be able to amplify the same region across different species.

3. **PCR amplification of diploid species.** DNA from *T. urartu*, *Ae. tauschii* and, *Ae. speltoides* are used to amplify the identified sequences.

4. **Sequence the amplicons**. The PCR products from the relative species are sequenced individually with capillary sequence.

5. **Alignment of the amplicons**. To search for variations between related species, the amplicons are aligned and the bases that are different across diploid species are used to design new primers, that should be genome specific in hexaploid wheat.

6. **Validation on nullisomic-tetrasomic lines**. Those research lines have been treated to remove one of their chromosome pairs. In the process, one of the homoeologue chromosomes is duplicated (four chromosomes, hence tetra). This lines are useful to evaluate the effect of a particular chromosome. In the case of genome-specific primer design, it is possible to evaluate if the primer is specific to the missing chromosome, as it will not amplify in the nullisomic-tetrasomic line. If the primer amplifies it means that it is not specific to the target chromosome (Figure 2.15).

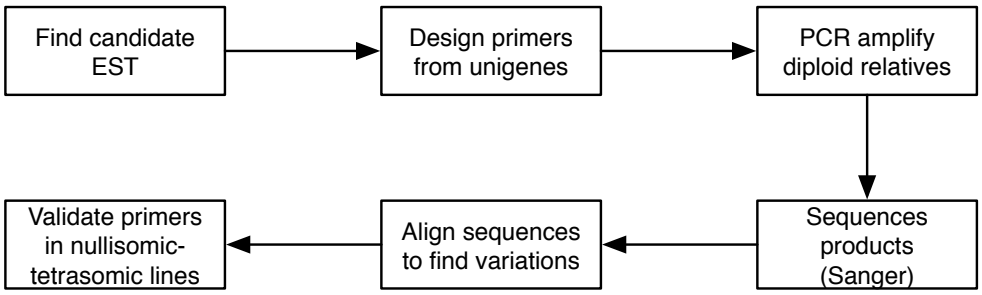


Figure 2.14: Previous process of primer design and validation

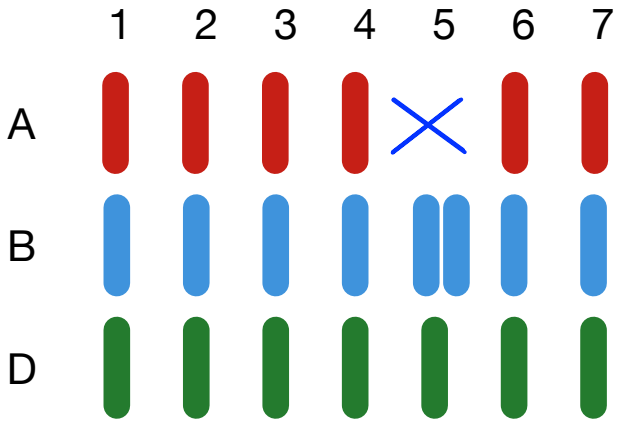


Figure 2.15: Nullisomic-tetrasomic lines. This example has chromosome 5A missing and 5B duplicated.

Even having a the sequence for each homoeologue, without an automated bioinformatic pipeline, the primer design was done manually, a slow, error-prone and, repetitive process. The steps require the use of several bioinformatics tools, but once I understood the primer design steps I decided to automate the process. Since designing genome-specific primers is a common task in wheat research and breeding, the community showed interest on the tool and I decided to refine it and make it open source. PolyMarker has been used successfully in several projects (Ramirez-Gonzalez et al., 2015c; Hubbard et al., 2015; Burt et al., 2015; King et al., 2015; Sollars et al., 2016) and it even allowed the novel use of KASP assays to validate long deletions in polyploids (Krasileva et al., submitted 2016).

As a common source of SNPs are gene models, designing primers directly from the sequence flanking the SNP may run over the intron-exon junctions, producing primers that will not amplify on genomic DNA. To be able to use DNA I had to identify on which hit the SNP was located, the internal coordinate and a mapping coordinate to the original

sequence. With this dual coordinate system I was able to design primers in the genome space, even when the origin was a transcript.

In order to be able to represent more than one base at the same tame, the IUAPC ambiguity codes (Cornish-Bowden, 1985) were useful in the development of PolyMarker. With an ambiguity code, the template for the search can contain the SNP. Also, the codes were useful when representing all the observed bases on each coordinate in the local alignment.

The ideas behind PolyMarker had been taken by other projects like the scripts described in Ma et al. (2015) and the corresponding web interface, GSP (Wang et al., 2016). Briefly, GSP does uses `blast` to search in a local database to find all the homoeologous regions and provides a diagram with the bases that are genome specific. It then allows the user to select a primer pair according to the constrains for their individual experiment, like product size. The advantage over PolyMarker is that it allows to pick arbitrary primers, at the cost of having a step for manual selection of the pair. Recently, LGC also developed a program (MAGICBOX) that require a SNP sequence, does the alignment and selects primers with a genome specific anchor. As PolyMarker, it produces a local alignment with the genome-specific bases (Curry et al., 2016) and a mask highlighting the variations providing the specificity to the primer. On personal communications in conferences I had found out that LGC, the company behind KASP, uses PolyMarker to design primers. Also Bayer has an in house implementation of the algorithm.

As the code is open source, anyone can see the implementation details and extend the code for different types of primers. A successful modification to PolyMarker was to be able to design primers to detect homozygous deletions with KASP assays, despite the fact that neither KASP or PolyMarker were designed for deletions. The modularity of the code permits to swap components with relatively little effort.

The current web interface of PolyMarker is limited to KASP assays, however the command line version is more flexible and has been used to design primers for PCR amplicons, capillary sequencing and on other organisms. However, to install the command requires a Linux machine and some knowledge on the command line.

PolyMarker is a tool that was originally designed to design the markers to validate the SNPs found in Chapter 3. Overall, PolyMarker provides an useful resource to the wheat community, as the primer design

process is now streamlined. PolyMarker is not restricted to wheat, it can be used on any polyploid systems, like the polyploids in the genus *Brassica*. As new references of wheat come available, PolyMarker should be updated to work with pseudo-molecules and the web interface updated accordingly. The source code of PolyMarker is open source and available on `https://github.com/TGAC/bioruby-polyploid-tools`.

Chapter 3

Genetic map of *Yr15* with RNA-Seq.

3.1 Background.

Wheat breeding programs aim to improve the wheat lines available for production. One of the traits desired in an elite line is the resistance to pathogens, such as*Puccinia striiformis* f. sp. *tritici* (PST), the fungi responsible of yellow rust. A source of resistance genes are introgressions from other species, such as *Triticum dicoccoides* (emmer, Figure 1.1). In the University of Sydney a collection of Near Isogenic Lines (NILs) with introgressions to several yellow rust resistance genes on a susceptible background were developed (Wellings and McIntosh, 1998). In this chapter the NIL for the *Yr15* locus is used to produce a mapping population to produce a mapping population, which when combined with mapping by sequencing approaches, results in improved diagnostic markers.

3.1.1 Segregation on F_2 populations.

Molecular markers can be used to select lines by testing if certain allele is present in a line, without the need to phenotype the given line. To find which regions are linked to a trait the use of F_2 mapping populations is a common practice, especially for major single gene traits. The population is produced by crossing two (usually homozygous) parents (P_1 and P_2) with different alleles, A/A (dominant, resistant if containing *Yr15*) and a/a (recessive, susceptible in our experiment). When the trait is domin-

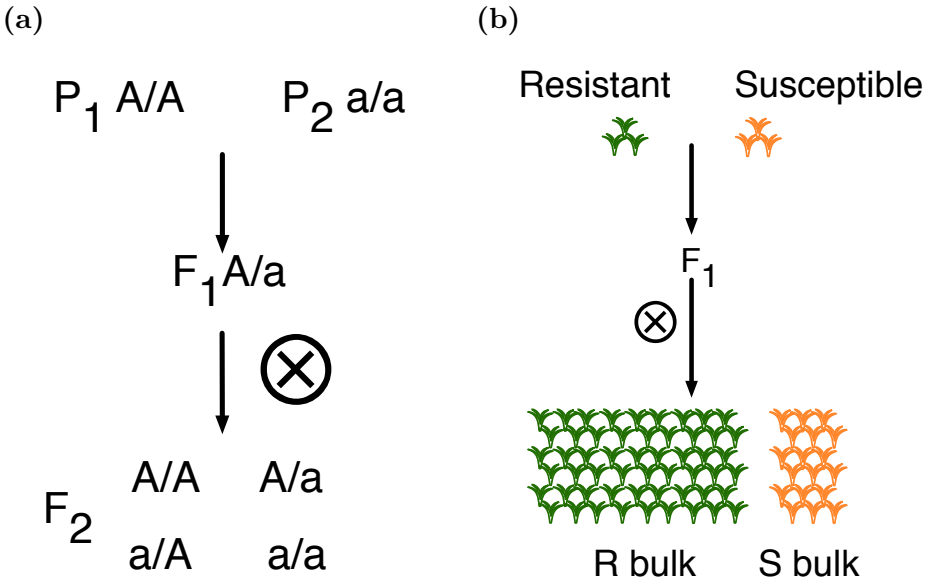


Figure 3.1: Alleles on F_2 population and Bulk Segregant Analysis. The $\otimes$ represent self-pollination. (a) The cross of two homozygous parents, P_1 and P_2, with a dominant and a recessive allele of a gene produces an heterozygous F_1. The F_1 crossed with itself produce a segregating F_2 population with a 1:2:1 ratio (A/A:A/a:a/a). The upper and lower cases represent dominant and recessive alleles, respectively. (b) Bulk Segregant Analysis consist on pooling DNA from the F_2 population. The DNA is mixed in bulks coming from plants with a shared phenotype. For a dominant resistance gene, an R sulk contains only resistant individuals (with A/A and A/a genotype) and, an S bulk with the susceptible individuals (with a/a genotype).

ant and has a Mendelian segregation, the F_1 population should exhibit the dominant trait, as it has a copy of each allele (A/a). The F_1 is then self-pollinated to produce and F_2 population which should segregate with a ratio of 1:2:1, dominant:heterozygous:recessive respectively. This generates a population with a phenotypic ratio of 3:1 (resistant:susceptible), since the effect of the recessive allele is masked by the dominant allele (Van Ooijen and Jansen 2013a; Figure 3.1a).

3.1.2 SNP calling

Bulk Segregant Analysis (BSA) consists on pooling the DNA of individuals from a segregating population with contrasting phenotypes (Michelmore et al., 1991) in a segregating population. By combining multiple independent individuals with similar phenotypes, one can identify regions which are over-represented or enriched in the corresponding bulks. Regions which are not linked to the trait of interest show up as heterozygous

in the bulks, whereas regions which are linked to the trait of interest will be enriched for either parental allele. Here one would expect an enrichment of the resistant allele A with respect to the susceptible allele a in the resistant bulk. Analogously, one would expect the absence of the resistant allele A in the susceptible bulk (Figure 3.1b). This approach can be used to identify SNPs using Next Generation Sequencing (NGS)-based methods, such as exome capture (Hodges et al., 2007), RNA-Seq (Pickrell et al., 2010), whole genome re-sequencing (Schneeberger et al., 2009), among others.

To find SNPs linked to the trait segregating in an F_2 population using NGS data there are several options. In organisms with a contiguous reference genome, a normalised count of the times each allele is observed is enough to find the region linked to the trait; this simple ratio is called SNP-Index (Takagi et al., 2013b). However, wheat is a polyploid organism, with an average identity between homoeologues of over 98%. Because of the high identity, reads coming from different homoeologues may map to the same position; and this problem is exacerbated in cases where a reference sequence for some of the homoeologues is absent. The Bulk Frequency Ratio (BFR) (Trick et al., 2012) methodology can work on organisms that have more than one pseudo genome and where not all of the genes, either homoeologues or paralogues, have been characterised independently; it works with a single reference by collapsing similar regions. Both methodologies rely on an enrichment of the alleles linked to the trait in the corresponding locus.

An example of homoeologous variants between two sub-genomes of wheat is the G>T variant at position 181; K in consensus (Figure 3.2). This variant will produce the same ambiguity code for both parental consensus sequences and can therefore be excluded. An example of real allelic varietal SNPs between the parental genotypes is exemplified by the G>A variant at position 184; R in consensus. These variants are distinguished by the presence in only one of the consensus sequences. The allelic SNPs are then examined further with the alignments of the bulks to identify the SNPs that are enriched on the resistant plants. The SNP index is the proportion of times an alternative allele is observed over the coverage at certain position, in the example the susceptible bulk has an SNP index of $1/8 = 0.125$ while the resistant bulk has an index of $6/8 = 0.75$ (Takagi et al., 2013b). The BFRs are then calculated by

dividing the SNP Index of the sample containing the target phenotype (resistance) over the sample without the trait (susceptible). For this example, it would be $0.75/0.125 = 6$. A high BFR suggests that the SNP is linked to the target trait (Trick et al., 2012). The implementation of the BFR analysis is detailed in Section 3.10.3 and the results on the F_2 population are discussed in Section 3.5.

To call SNPs from RNA-Seq, a reference transcriptome rather than a reference genome sequence is used as target to align the reads. The UniGenes database, from NCBI, contains the known genes of each species with all the variations of each gene automatically collapsed and represented with the longest available cDNA (Pontius et al., 2002). The UCW gene set described in Krasileva et al. (2013) contains 94,177 models from tetraploid and hexaploid wheat, assembled and phased to separate different homoeologues. Both gene sets complement each other, however, the UCW gene models should provide an improved alignment, since the different homoeologues have not merged in a single model - a possible side effect of the UniGene pipeline.

3.1.3 *In Silico* mapping.

There are several layers of information that can be used to add a context to the SNPs. When the SNPs are called from genes like the UniGenes (Pontius et al., 2002) or the UCW gene models (Krasileva et al., 2013), the location of the genes can be assigned by aligning them to a genomic reference, even if it is fragmented. A source to get the order of the scaffolds are previously published genetic maps, such as the one described in Wang et al. (2014), which has the sequence of the markers available. The markers and the genes can be aligned to the scaffolds with a high identity cutoff (over 98%), to avoid them being assigned to a homoeologue or paralogue on a different chromosome. The practice of using genetic maps to sort genomic sequence and produce pseudo-chromosomes is common in genome wide projects, and is usually performed with *ad-hoc* tools (Tang et al., 2015). The highly fragmented state of the CSS assembly prevents the use of genetic maps to produce pseudo-molecules, as those maps which are currently available do not have enough resolution. However, they are dense enough to sort the scaffolds in bins when several markers map to the same location. In this way, it is possible to use the scaffolds as a proxy to map the genes to their genetic position (Figure

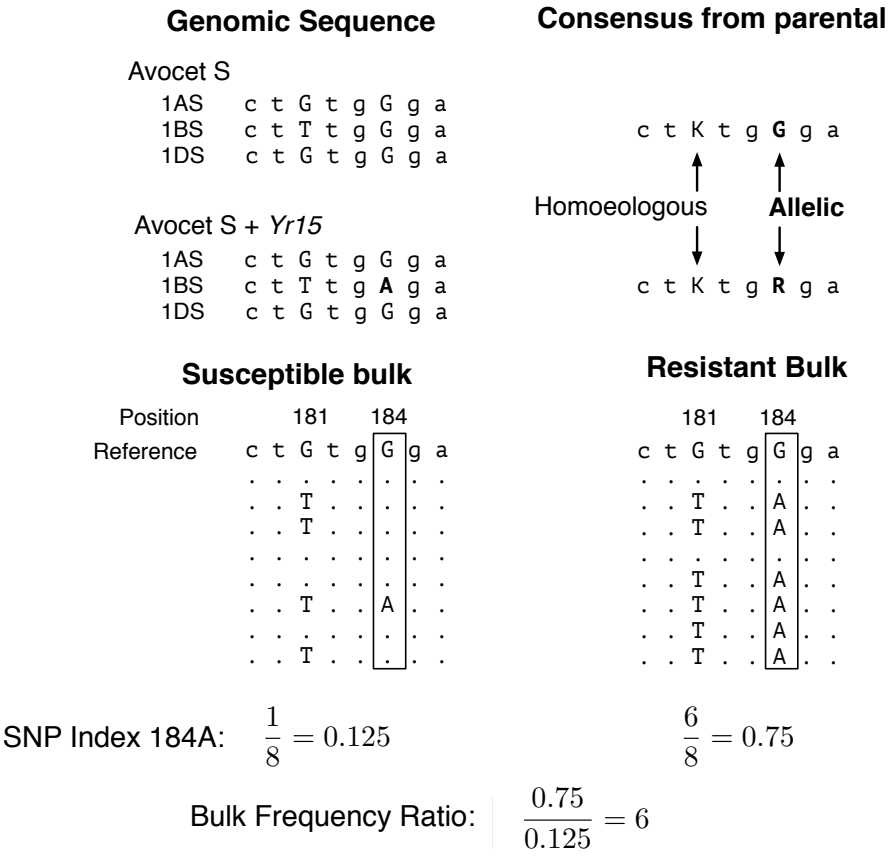


Figure 3.2: BFR formula. Illustration of a non-informative homoeologous SNP (G181T) present in both parental lines, and an informative allelic SNP (G184A), only present in the resistant progenitor Avocet S + Yr15. The consensus sequences from the parental genotypes include this information in the form of ambiguity codes (K and R, respectively). In the bulks, the individual reads align across the reference sequence, with matches indicated by dots, and polymorphisms at positions 181 and 184 indicated by the corresponding nucleotide variants. The SNP index is calculated as the frequency of the informative allelic SNP in each bulk. The Bulk Frequency Ratio is the quotient of the resistant and susceptible bulk SNP Indexes. Figure previously published in Ramirez-Gonzalez et al. (2015b).

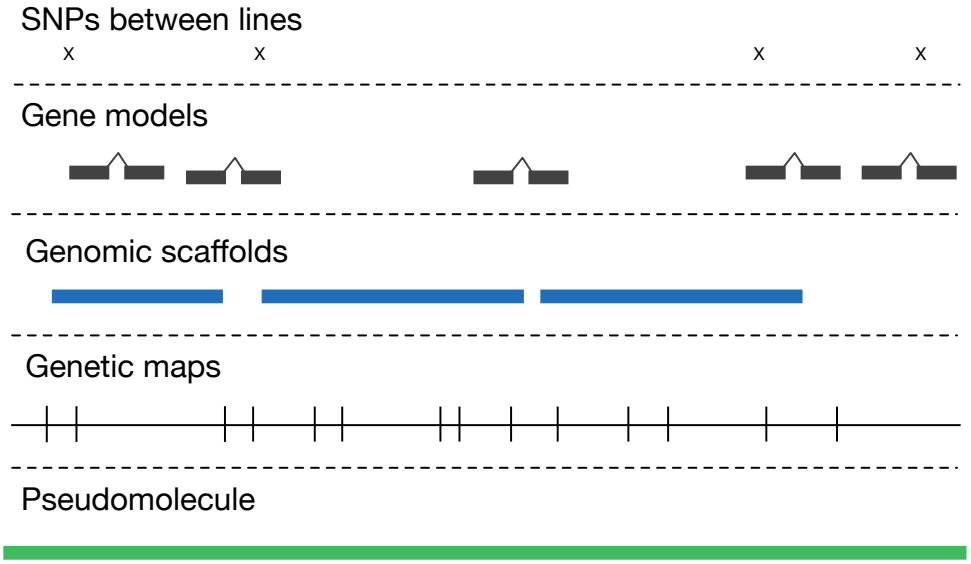


Figure 3.3: Layers of information to do *In Silico* mapping. SNPs are called from gene models. The genes and markers from genetic maps are aligned to scaffolds. The order of the markers in a genetic map can be used to sort the scaffolds.

3.3). The results of mapping the genes with SNPs to the CSS assembly and the genetic map are described in Section 3.6. For a longer description of resources available for wheat see Section 1.4.

Finally, the best candidate SNPs were selected to produce a genetic map which lead to a triplet of markers diagnostic for the target locus.

The steps described in this chapter were first published in Ramirez-Gonzalez et al. (2015b) and the results of this chapter are published in Ramirez-Gonzalez et al. (2015c).

3.2 Mapping population.

The population was developed by crossing the resistant line Avocet + *Yr15* (*Yr15*) (Wellings and McIntosh, 1998), Figure 3.4a, to the susceptible line Avocet S (AVS), Figure 3.4b. *Yr15* is a NIL of a 6th generation Back-cross (BC) and the AVS background is highly susceptible to yellow rust, hence the resistance is conferred by the *Yr15* locus. F_2 seeds from three independent F_1 plants where sown and tissue was collected before fungal inoculation to avoid the effect of the disease resistance response on gene expression. Sampling after inoculation could have led to associations in the bulks due to expression of genes downstream of

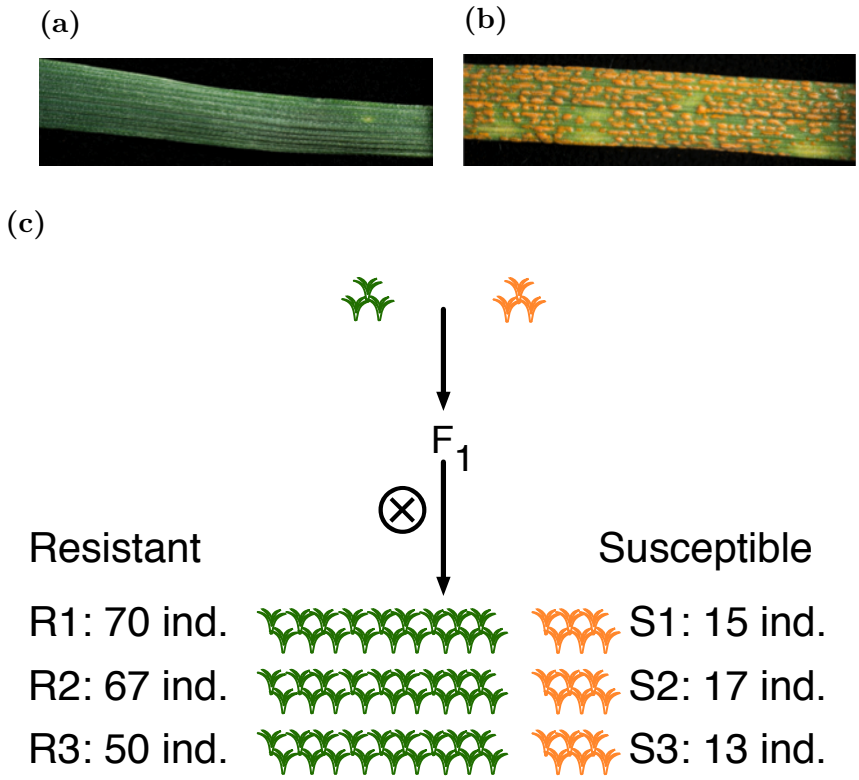


Figure 3.4: Avocet + *Yr15* F_2 mapping population. Response of (a) Avocet + *Yr15* and (b) Avocet when inoculated with *Puccinia striiformis* f. sp. *tritici* at the three leaf stage. (c) The phenotype of the F_2 population was used to produce 6 bulks, 3 resistant and 2 susceptible. The RNA was pooled in bulks accordingly. Adapted from (Ramirez-Gonzalez et al., 2015c)

Yr15 and not due to the gene itself. Seedlings were challenged at the three leaf stage as it is known that *Yr15* confers resistance in seedlings (Gerechter-Amitai et al., 1989). The expected segregation of a F_2 population is 3:1 (resistant:susceptible), since *Yr15* is a dominant gene. From the 232 plants in the F_2 population that germinated, 187 were resistant and 45 were susceptible, which deviates slightly from the expected ratio ($\chi^2 = 0.049$). Segregation distortion has been shown for the same *Yr15* donor (Randhawa et al., 2009), however the decreased number of susceptible plants can be explained by escapes in the virulence essays (i.e. plants scored as resistant without the *Yr15* locus). For this study, we extracted DNA from individual plants in the F_2 population and we bulked RNA on 6 different bulks: 3 resistant and 3 susceptible (Figure 3.4c).

Table 3.1: Arrangement and number of sequenced base pairs per sample.

Library	name	Bar code	Lane	Reads ($\times 10^8$ bp)
LIB1715	Bulk R1	ATCACG	1	0.77
LIB1716	Bulk R2	TAGCTT	1	1.20
LIB1717	Bulk R3	ACTTGA	2	0.96
LIB1718	Bulk S1	GGCTAC	2	1.64
LIB1719	Bulk S2	CGTACG	2	1.49
LIB1720	Bulk S3	GTGGCC	1	1.88
LIB1721	AvocetS	N/A	3	4.13
LIB1722	AvocetS + *Yr15*	N/A	4	3.99

3.3 Sequencing and mapping.

RNA-Seq was used as a reduced representation method, and thus avoided sequencing the non-coding regions. This effectively reduces the search space, which is especially important in a species with a genome as rich in repeat content as wheat. The sequencing of the bulks and the parents was done on a single Illumina Hi-Seq2000. The bulks were multiplexed and sequenced on a third of a lane each, as shown on Table 3.1. To ensure that quality of sequencing, `fastqc-0.10` (Babraham Bioinformatics, 2012) was run with its default parameters for each of the FASTQ files. The GC content was around 52% in all the samples (Appendix B.2), which is as expected as the sample should be of coding regions, and for wheat the reported GC content in genes is around 55%. The quality of the reads is fairly consistent, in general dropping after base 80 across samples (Appendix B.1).

When the analysis was started, the draft genome and the corresponding annotation had not been released yet, hence gene models were used instead of a genome reference. All the samples were aligned to the UniGenes v60 (56,954 genes) and the gene models from UCW (Krasileva et al., 2013) using `BWA 0.5.9` (Li and Durbin, 2009). The alignment showed that few genes were very highly expressed, however, there was still sufficient coverage of over 20x in *Yr15* across 22,107 and 36,808 genes, on the UniGenes and the UCW gene set, respectively. Both gene sets performed similarly in terms of the percentage of genes with reads and percentage of aligned reads. The percentage of genes with a coverage of at least $20x$ is 45% and 39% for AVS and *Yr15*, irrespective of the reference gene set chosen (Figure 3.5a). Since each individual bulk has a

Table 3.2: Number of genes with a coverage over 20x, 10x and at least one read (>0x).

		Bulks						Bulk mixes				Progenitors	
Coverage	Reference	R1	R2	R3	S1	S2	S3	R1+R2	S1+S2	R1+R2+R3	S1+S2+S3	*Yr15*	AVS
20x	UCW	16,434	27,871	27,223	32,287	28,669	34,898	33,968	41,019	40,985	47,507	36,808	42,248
		17%	30%	29%	34%	30%	37%	36%	44%	44%	50%	39%	45%
	UniGene v60	9,643	16,182	15,222	19,549	17,397	20,567	20,219	25,270	24,598	29,052	22,107	25,842
		17%	28%	27%	34%	31%	36%	36%	44%	43%	51%	39%	45%
10x	UCW	27,371	38,282	37,777	42,658	38,999	44,610	43,266	49,473	49,182	54,781	46,356	50,760
		29%	41%	40%	45%	41%	47%	46%	53%	52%	58%	49%	54%
	UniGene v60	16,201	22,948	22,130	26,200	24,130	26,914	26,318	30,579	29,857	33,557	28,044	31,095
		28%	40%	39%	46%	42%	47%	46%	54%	52%	59%	49%	55%
>0x	UCW	68,302	72,484	72,957	74,694	73,290	75,201	74,397	77,093	76,715	78,796	76,275	77,080
		73%	77%	77%	79%	78%	80%	79%	82%	81%	84%	81%	82%
	UniGene v60	40,717	42,489	42,595	43,625	43,059	43,748	43,393	44,655	44,364	45,392	43,732	44,596"
		71%	75%	75%	77%	76%	77%	76%	78%	78%	80%	77%	78%

lower coverage, the susceptible and resistant reads were merged *in silico* as: (i) susceptible bulks 1 with 2 (S1+S2) and resistant bulks 1 with 2 (R1+R2) and (ii) all the susceptible (S1+S2+S3) and resistant bulks (R1+R2+R3). The merged samples increased the percentage of genes with coverage over 20x to 44% and 50% in the resistant and susceptible bulks (Table 3.2), which is close to the coverage from the progenitors. We treated bulk 3 slightly differently since these bulks included a few lines which were borderline with respect to their phenotype. Therefore exclusion of bulk 3 plants in the S1+S2 and R1+R2 bulk would provide the "cleanest" possible data, whereas inclusion in the second set of bulks would allow us to evaluate the effect of possible noise within the system.

3.4 SNP Calling

The SNP calling was done on positions with a coverage of at least $20x$ on the progenitor lines against the gene reference. The AVS progenitor had roughly 3% more genes with polymorphisms than *Yr15*, consistent with the difference in coverage, suggesting that with a higher coverage we could recover more SNPs from *Yr15*. The UniGenes have a higher number of SNPs because the University of California Wheat (UCW) gene models have a higher number of monomorphic genes when compared to the UniGenes (Figure 3.5b; Table 3.3). The difference in the number of relative monomorphic SNPs between reference can be explained by the fact that in the UniGenes set many homologous might have been collapsed into a single representative sequence, whereas the UCW gene set is homoeologue-specific. Therefore, mapping to the correct homoeologue is improved in the UCW gene set over the UniGenes.

Both gene sets were derived from varieties different to AVS and are likely to be incomplete, hence we set a low threshold of at least 20% of the observed nucleotides on any position to call a SNP. To represent cases where more than one consensus base is called we use International Union of Pure and Applied Chemistry (IUPAC) codes (Cornish-Bowden (1985); Section 1.3; Figure 3.2). To focus the analysis on informative SNPs, the common varietal SNPs and variations between homoeologues were removed by finding cases where the consensus call on both progenitors was the same. The SNPs that are unique to a single parental were

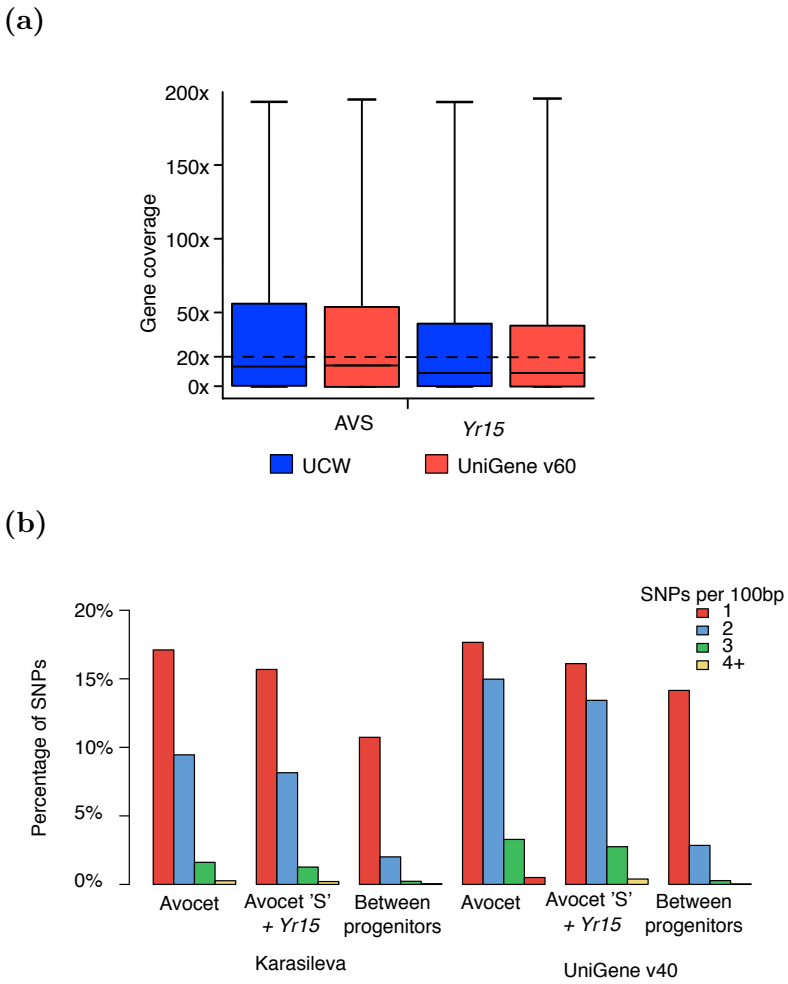


Figure 3.5: Coverage and SNPs between progenitors. (a) Box plot distribution of the gene coverage of the parent reads (AVS and *Yr15*) across the UCW (blue) and the UniGene (red) gene models. The dashed line represents the 20x minimum coverage required for SNP calling. The full line represents the average coverage across all gene models. (b) Percentage of genes exhibiting SNPs across references. The number of Single Nucleotide Polymorphism (SNP)s between the parent reads and the corresponding references was calculated (per 100 bp, rounded). The 'between-parents' category corresponds to putative SNPs when comparing the consensus sequence between AVS and *Yr15* Adapted from Ramirez-Gonzalez et al. (2015c)

Table 3.3: Count of SNPs per 100 bp on genes with at least 20x coverage.

SNPs per 100bp	UCW			UniGene v60		
	AVS	AVS+ *Yr15*	Between progenitors	AVS	AVS+ *Yr15*	Between progenitors
0	67, 389 71.6%	70,338 74.7%	81,921 87.0%	36,210 63.6%	38,339 67.3%	47,097 82.7%
1	16,111 17.1%	14,770 15.7%	10,107 10.7%	10,058 17.7%	9,175 16.1%	8,061 14.2%
2	8,904 9.5%	7,676 8.2%	1,893 2.0%	8,529 15.0%	7,648 13.4%	1,621 2.9%
3	1,517 1.6%	1,192 1.3%	215 0.2%	1,870 3.3%	1,568 2.8%	59 0.3%
4+	253 0.3%	198 0.2%	38 0.0%	287 0.5%	224 0.4%	16 0.0%

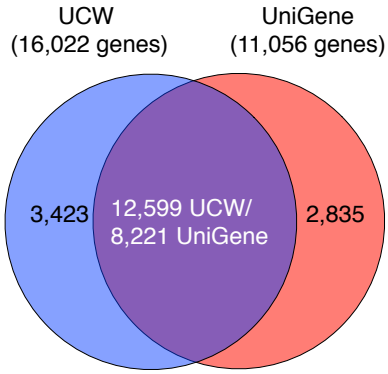


Figure 3.6: Gene models with putative SNPs in common between the UCW and UniGenes reference. The intersection represents the genes that are common in both sets. Adapted from Ramirez-Gonzalez et al. (2015c)

examined in detail. There are 66,426 putative SNPs across 16,022 (17%) UCW genes and 52,262 SNPs on 11,056 UniGenes (19.4%; Figure 3.6).

The high number of genes with SNPs was unexpected as a BC6 NIL used for a F_2 mapping population expects to have less than 1% of the genetic background segregating. Both sets of gene models were aligned with BLAT (Kent, 2002) to the Chinese Spring Chromosome arm survey sequence (CSS) assembly (Mayer et al., 2014); the alignment resulted on 80,031 (85.0%) UCW gene models and 41,118 (72.2%) UniGenes assigned to a chromosome arm (Table 3.4). The SNPs found in the mapped genes are evenly distributed across all the chromosomes (see Section 3.6), suggesting that the AVS (John Innes Centre (JIC), UK) used as parent in the F_2 is different to the AVS used for the *Yr15* NIL development (University of Sydney, Australia).

To confirm that the AVS seed stocks from JIC are distinct to the stocks in Sydney, DNA from both stocks was procured and compared

Table 3.4: Number of genes with SNPs assigned to the wheat chromosome arm CSS scaffolds (Mayer et al., 2014) using the best hit from BLAT (Kent, 2002)

Wheat Chromosome Arm	UCW (94,177)	UniGene v60 (56,954)	Total (151,131)
1AL	3,251 (3.45%)	1,404 (2.47%)	4,655 (3.08%)
1AS	1,366 (1.45%)	560 (0.98%)	1,926 (1.27%)
1BL	2,610 (2.77%)	1,280 (2.25%)	3,890 (2.57%)
1BS	1,487 (1.58%)	693 (1.22%)	2,180 (1.44%)
1DL	997 (1.06%)	1,057 (1.86%)	2,054 (1.36%)
1DS	753 (0.80%)	687 (1.21%)	1,440 (0.95%)
2AL	3,491 (3.71%)	1,460 (2.56%)	4,951 (3.28%)
2AS	2,305 (2.45%)	974 (1.71%)	3,279 (2.17%)
2BL	3,658 (3.88%)	1,546 (2.71%)	5,204 (3.44%)
2BS	2,790 (2.96%)	1,139 (2.00%)	3,929 (2.60%)
2DL	1,098 (1.17%)	1,069 (1.88%)	2,167 (1.43%)
2DS	796 (0.85%)	833 (1.46%)	1,629 (1.08%)
3AL	2,135 (2.27%)	978 (1.72%)	3,113 (2.06%)
3AS	1,543 (1.64%)	718 (1.26%)	2,261 (1.50%)
3B	6,559 (6.96%)	2,839 (4.98%)	9,398 (6.22%)
3DL	915 (0.97%)	938 (1.65%)	1,853 (1.23%)
3DS	412 (0.44%)	450 (0.79%)	862 (0.57%)
4AL	3,393 (3.60%)	1,335 (2.34%)	4,728 (3.13%)
4AS	2,011 (2.14%)	817 (1.43%)	2,828 (1.87%)
4BL	2,119 (2.25%)	898 (1.58%)	3,017 (2.00%)
4BS	1,946 (2.07%)	892 (1.57%)	2,838 (1.88%)
4DL	1,069 (1.14%)	945 (1.66%)	2,014 (1.33%)
4DS	800 (0.85%)	699 (1.23%)	1,499 (0.99%)
5AL	2,640 (2.80%)	1,132 (1.99%)	3,772 (2.50%)
5AS	963 (1.02%)	407 (0.71%)	1,370 (0.91%)
5BL	5,324 (5.65%)	1,943 (3.41%)	7,267 (4.81%)
5BS	1,360 (1.44%)	591 (1.04%)	1,951 (1.29%)
5DL	2,067 (2.19%)	1,688 (2.96%)	3,755 (2.48%)
5DS	620 (0.66%)	614 (1.08%)	1,234 (0.82%)
6AL	2,397 (2.55%)	896 (1.57%)	3,293 (2.18%)
6AS	2,285 (2.43%)	936 (1.64%)	3,221 (2.13%)
6BL	1,564 (1.66%)	820 (1.44%)	2,384 (1.58%)
6BS	1,308 (1.39%)	731 (1.28%)	2,039 (1.35%)
6DL	1,399 (1.49%)	1,050 (1.84%)	2,449 (1.62%)
6DS	870 (0.92%)	680 (1.19%)	1,550 (1.03%)
7AL	1,918 (2.04%)	849 (1.49%)	2,767 (1.83%)
7AS	1,717 (1.82%)	764 (1.34%)	2,481 (1.64%)
7BL	1,592 (1.69%)	776 (1.36%)	2,368 (1.57%)
7BS	1,239 (1.32%)	713 (1.25%)	1,952 (1.29%)
7DL	2,040 (2.17%)	1,301 (2.28%)	3,341 (2.21%)
7DS	1,224 (1.30%)	1,016 (1.78%)	2,240 (1.48%)
Assigned	80,031 (84.98%)	41,118 (72.20%)	121,149 (80.16%)

Table 3.5: Total number of SNPs scored in parents, individual bulks and in silico merged bulks.

Gene set	$\frac{R1}{S1}$	$\frac{R2}{S2}$	$\frac{R3}{S3}$	$\frac{R1+R2}{S1+S2}$	$\frac{R1+R2+R3}{S1+S2+S3}$	SNPs in parents
UCW	16,269 24.49%	29,703 44.72%	31,891 48.01%	44,224 66.58%	64,522 97.13%	66,426
UniGene v60	15,261 29.20%	25,143 48.11%	24,548 46.97%	35,698 68.31%	49,738 95.17%	52,262

with the iSelect 90k wheat SNP chip. Between two independent AVS seeds from JIC only 58 out of 71,972 (0.08%) valid assays were polymorphic. Nonetheless, there are over 5,000 ($> 7.5\%$) assays with polymorphisms between JIC-AVS and AVS from Sydney. The difference was not expected originally, but considering that the AVS seeds are coming from different stocks and the fact that in both countries commercial varieties with the same name had been released, it is not surprising.

3.5 Bulk Frequency Ratios

The objective was to find the SNPs enriched (or depleted) in each bulk and hence linked to the phenotype. SNPs originating from *Yr15* would be expected to be linked to resistance whereas those from AVS to susceptibility in the segregating population. Across individual bulks, it was possible to score between 15,261 (24.5%) and 31,891(48.0%) SNPss across both reference sets. On the *in silico* mixes, over 95% of SNPs where scored (Table 3.5), suggesting that the coverage of individual bulks is not enough to score all the SNPs. The scoring was done with the Bulk Frequency Ratio (Trick et al. 2012;Figure 3.2; Section 3.10.3), which has a value that increases as the *Yr15* allele is observed more times relatively to the AVS allele.

When increasing the minimum BFR threshold, enrichment of SNPs was observed in the short arm of the group 1 chromosomes (1S). Without taking into account the BFR, 3.6% of the SNPs are located in the 1S group, similar to the number of SNPs located in other groups 3.4. However, when increasing the threshold (between $BFR > 5$ and $BFR > 7$) the relative number of SNPs in group 1S increases. After $BFR > 7$ the gains in relative enrichment only improves marginally, but the number of called SNPs is reduced (Table 3.6; Figure 3.7). For that reason, SNPs

Table 3.6: SNPs in chromosome group 1S vs total number of SNPs with a minimum BFR from 0 to 10. AVS: SNPs coming from Avocet S. *Yr15*: SNPs coming from Avocet + *Yr15*.

Min BFR	Gene Set	R1/S1 *Yr15*	R1/S1 AVS	R2/S2 *Yr15*	R2/S2 AVS	R3/S3 *Yr15*	R3/S3 AVS	S1+2/ R1+2 *Yr15*	S1+2/ R1+2 AVS	S1+S2+S3/ R1+R2+R3 *Yr15*	S1+S2+S3/ R1+R2+R3 AVS
0	UCW	308/8,049 (3.83%)	305/8,220 (3.71%)	505/14,121 (3.58%)	556/15,582 (3.57%)	532/14,875 (3.58%)	623/17,016 (3.66%)	670/18,760 (3.57%)	885/25,464 (3.48%)	860/24,026 (3.58%)	1,505/40,496 (3.72%)
	UniGene v60	307/7,823 (3.92%)	299/7,438 (4.02%)	428/12,409 (3.45%)	421/12,734 (3.31%)	427/12,050 (3.54%)	415/12,498 (3.32%)	536/15,672 (3.42%)	595/20,026 (2.97%)	712/19,358 (3.68%)	901/30,380 (2.97%)
1	UCW	214/4,415 (4.85%)	194/4,108 (4.72%)	325/7,603 (4.27%)	314/7,374 (4.26%)	365/7,920 (4.61%)	415/8,850 (4.69%)	426/10,122 (4.21%)	494/12,185 (4.05%)	539/13,037 (4.13%)	842/19,466 (4.33%)
	UniGene v60	207/4,474 (4.63%)	194/3,630 (5.34%)	269/6,649 (4.05%)	269/6,193 (4.34%)	279/6,511 (4.29%)	272/6,436 (4.23%)	329/8,704 (3.78%)	369/9,343 (3.95%)	446/10,860 (4.11%)	541/14,226 (3.80%)
2	UCW	92/651 (14.13%)	75/671 (11.18%)	142/1,372 (10.35%)	111/1,101 (10.08%)	147/1,162 (12.65%)	149/1,411 (10.56%)	167/1,324 (12.61%)	163/1,478 (11.03%)	194/1,370 (14.16%)	207/1,765 (11.73%)
	UniGene v60	77/568 (13.56%)	58/527 (11.01%)	101/1,017 (9.93%)	81/720 (11.25%)	105/775 (13.55%)	84/867 (9.69%)	122/991 (12.31%)	116/973 (11.92%)	145/1,030 (14.08%)	132/1,210 (10.91%)
3	UCW	78/299 (26.09%)	45/295 (15.25%)	118/646 (18.27%)	70/409 (17.11%)	123/577 (21.32%)	85/494 (17.21%)	145/673 (21.55%)	98/563 (17.41%)	168/768 (21.88%)	122/665 (18.35%)
	UniGene v60	65/254 (25.59%)	26/186 (13.98%)	87/499 (17.43%)	54/294 (18.37%)	93/379 (24.54%)	48/315 (15.24%)	107/525 (20.38%)	66/379 (17.41%)	133/617 (21.56%)	78/489 (15.95%)
4	UCW	75/232 (32.33%)	28/160 (17.50%)	109/484 (22.52%)	44/217 (20.28%)	105/416 (25.24%)	44/246 (17.89%)	134/539 (24.86%)	53/277 (19.13%)	149/640 (23.28%)	64/323 (19.81%)
	UniGene v60	63/192 (32.81%)	17/104 (16.35%)	83/390 (21.28%)	29/155 (18.71%)	82/288 (28.47%)	29/173 (16.76%)	104/431 (24.13%)	40/214 (18.69%)	127/519 (24.47%)	29/266 (10.90%)
5	UCW	69/202 (34.16%)	19/108 (17.59%)	95/416 (22.84%)	33/138 (23.91%)	96/354 (27.12%)	23/143 (16.08%)	127/477 (26.62%)	28/175 (16.00%)	140/580 (24.14%)	42/222 (18.92%)
	UniGene v60	58/163 (35.58%)	11/70 (15.71%)	76/337 (22.55%)	14/102 (13.73%)	70/228 (30.70%)	20/112 (17.86%)	100/389 (25.71%)	23/146 (15.75%)	118/469 (25.16%)	21/178 (11.80%)
6	UCW	65/179 (36.31%)	12/85 (14.12%)	86/380 (22.63%)	22/98 (22.45%)	87/299 (29.10%)	11/94 (11.70%)	122/429 (28.44%)	21/130 (16.15%)	126/514 (24.51%)	29/165 (17.58%)
	UniGene v60	57/151 (37.75%)	7/48 (14.58%)	73/300 (24.33%)	6/71 (8.45%)	65/191 (34.03%)	13/84 (15.48%)	98/358 (27.37%)	20/122 (16.39%)	115/439 (26.20%)	16/143 (11.19%)
7	UCW	58/161 (36.02%)	11/73 (15.07%)	77/340 (22.65%)	13/74 (17.57%)	73/248 (29.44%)	7/69 (10.14%)	116/393 (29.52%)	20/111 (18.02%)	114/468 (24.36%)	22/143 (15.38%)
	UniGene v60	56/132 (42.42%)	4/37 (10.81%)	68/273 (24.91%)	5/58 (8.62%)	60/171 (35.09%)	9/64 (14.06%)	94/334 (28.14%)	18/103 (17.48%)	113/412 (27.43%)	16/124 (12.90%)
8	UCW	58/149 (38.93%)	10/62 (16.13%)	68/310 (21.94%)	12/59 (20.34%)	66/214 (30.84%)	6/56 (10.71%)	104/359 (28.97%)	17/102 (16.67%)	108/429 (25.17%)	16/119 (13.45%)
	UniGene v60	55/126 (43.65%)	3/33 (9.09%)	64/255 (25.10%)	5/50 (10.00%)	55/150 (36.67%)	9/55 (16.36%)	91/313 (29.07%)	14/89 (15.73%)	105/376 (27.93%)	15/108 (13.89%)
9	UCW	54/135 (40.00%)	8/53 (15.09%)	63/289 (21.80%)	8/51 (15.69%)	61/182 (33.52%)	5/49 (10.20%)	100/331 (30.21%)	15/91 (16.48%)	100/387 (25.84%)	13/106 (12.26%)
	UniGene v60	53/117 (45.30%)	1/30 (3.33%)	62/244 (25.41%)	5/46 (10.87%)	50/136 (36.76%)	9/48 (18.75%)	88/291 (30.24%)	13/83 (15.66%)	97/345 (28.12%)	12/99 (12.12%)
10	UCW	52/126 (41.27%)	8/50 (16.00%)	62/279 (22.22%)	8/50 (16.00%)	56/165 (33.94%)	4/45 (8.89%)	96/309 (31.07%)	14/82 (17.07%)	91/355 (25.63%)	13/100 (13.00%)
	UniGene v60	50/105 (47.62%)	1/28 (3.57%)	60/226 (26.55%)	5/39 (12.82%)	43/119 (36.13%)	7/45 (15.56%)	85/272 (31.25%)	13/82 (15.85%)	92/318 (28.93%)	12/97 (12.37%)

with a $BFR > 6$ were selected for further validation. The method described by Trick et al. (2012) was extended to include cases where there is a complete lack of coverage in one of the samples ($BFR = \infty$), which is an ideal case where the linkage between the SNP and the phenotype is perfect. A total of 1,582 SNPs across 1,173 genes had a $BFR > 6$.

3.6 *In silico* mapping

From the mapped SNPs with a $BFR > 6$, 872 of 1,582 ($\sim 60\%$) were assigned to the chromosomes in group 1 of hexaploid wheat, being the only group with more than 4% of the SNPs assigned to it (Table 3.7). From group 1, the B genome contained the higher proportion of SNPs mapped (54%), having 255 (54%) and 214 (46%) assigned to the long and short arms respectively (Figure 3.8). These results are expected since previous studies have located *Yr15* near the centromere in the short arm of chromosome 1B and the *Yr15* introgression contains regions from the long and short arm from *T. dicoccoides* (Murphy et al., 2009; Peng et al., 2000; Sun et al., 1997).

The CSS assembly was used as a common reference between the reference genes and the 40,266 SNP markers published at the time (Wang et al., 2014) to locate the SNPs with a $BFR > 6$ (including $BFR = \infty$) in a genomic position (Figures 3.9, 3.10). From the 1,582 SNPS across 1,173 genes, only 678 SNPs (43%, 474 genes) were successfully located in the genetic map. Since the CSS assembly is quite fragmented, the low percentage of located SNPs can be because not all candidate SNPs had a corresponding scaffold that has at least one of the 40,266 markers in the genetic map. Even if the number of located SNPs was not enough to give a position for over 50% of the SNPs from the parental line, the resolution of the genetic position SNPs that were assigned improved over just having the chromosome arm information from the CSS assembly. The mapping position further confirmed an enrichment of SNPs near the centromere of chromosome 1B with 325 out of 678 SNPs. Furthermore, 311 of those where located within an interval of 30cM (Figures 3.10b, 3.9a).

Studies in diploid organisms using QTL-Seq (Takagi et al., 2013a) or other NGS-enabled genetic approaches (James et al., 2013) have shown smooth curves with a defined peak in the region linked to the studied

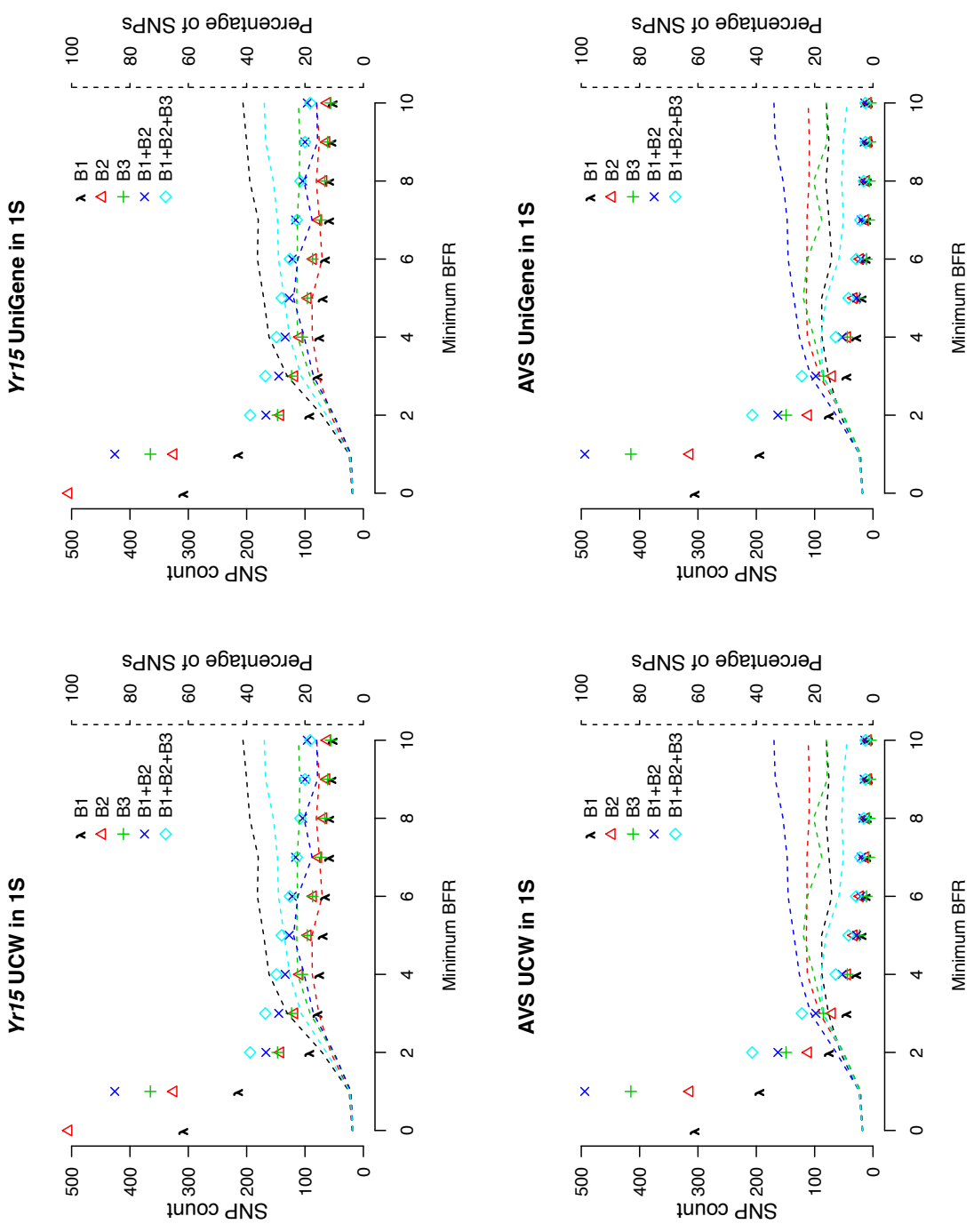


Figure 3.7: Effect of BFR threshold on the number of SNPs across the short arm of chromosome group 1. Count of SNPs with across different BFR threshold, in the short arm of chromosome group 1. Top row: SNPs from AVS+ *Yr15*; Bottom row: AVS; Left: UCW gene models: Right: UniGene v60. On dotted lines, the percentage of SNPs that map to the group 1S. The symbols represent the count of SNPs. The count of SNPs from AVS decreases faster than the count of SNPs from AVS+ *Yr15* across the bulks. Figure previously published in Ramirez-Gonzalez et al. (2015c).

Table 3.7: SNP and genes with BFR > 6 mapping to each of the chromosomes from the CSS assemblies. The chromosome assignment on the "Genetically mapped" column correspond to the map published in Wang et al. (2014)).

Reference	CSS assemblies								Genetically mapped							
Chromosome	UCW gene models				UniGene v60				UCW gene models				UniGene v60			
arm	SNPs		Genes		SNPs		Genes		SNPs		Genes		SNPs		Genes	
1AL	113	13.15%	79	12.29%	78	10.79%	50	9.43%	14	1.63%	8	1.24%	7	0.97%	4	0.75%
1AS	26	3.03%	21	3.27%	20	2.77%	17	3.21%	42	4.89%	32	4.98%	38	5.26%	28	5.28%
1BL	157	18.28%	110	17.11%	98	13.55%	64	12.08%	60	6.98%	35	5.44%	36	4.98%	23	4.34%
1BS	120	13.97%	74	11.51%	94	13.00%	44	8.30%	127	14.78%	80	12.44%	102	14.11%	46	8.68%
1DL	30	3.49%	21	3.27%	58	8.02%	47	8.87%	2	0.23%	2	0.31%	4	0.55%	4	0.75%
1DS	40	4.66%	25	3.89%	38	5.26%	24	4.53%	12	1.40%	6	0.93%	8	1.11%	5	0.94%
2AL	22	2.56%	20	3.11%	14	1.94%	12	2.26%	9	1.05%	8	1.24%	7	0.97%	5	0.94%
2AS	11	1.28%	11	1.71%	10	1.38%	7	1.32%	9	1.05%	9	1.40%	2	0.28%	2	0.38%
2BL	17	1.98%	15	2.33%	18	2.49%	17	3.21%	7	0.81%	5	0.78%	4	0.55%	4	0.75%
2BS	11	1.28%	10	1.56%	12	1.66%	7	1.32%	13	1.51%	12	1.87%	7	0.97%	7	1.32%
2DL	2	0.23%	2	0.31%	15	2.07%	10	1.89%	1	0.12%	1	0.16%	3	0.41%	2	0.38%
2DS	0	0.00%	0	0.00%	5	0.69%	3	0.57%	0	0.00%	0	0.00%	0	0.00%	0	0.00%
3AL	7	0.81%	7	1.09%	2	0.28%	2	0.38%	2	0.23%	2	0.31%	1	0.14%	1	0.19%
3AS	1	0.12%	1	0.16%	4	0.55%	4	0.75%	0	0.00%	0	0.00%	0	0.00%	0	0.00%
3B	31	3.61%	26	4.04%	28	3.87%	24	4.53%	0	0.00%	0	0.00%	0	0.00%	0	0.00%
3BL	0	0.00%	0	0.00%	0	0.00%	0	0.00%	9	1.05%	7	1.09%	4	0.55%	4	0.75%
3BS	0	0.00%	0	0.00%	0	0.00%	0	0.00%	2	0.23%	2	0.31%	5	0.69%	5	0.94%
3DL	7	0.81%	6	0.93%	2	0.28%	2	0.38%	1	0.12%	1	0.16%	0	0.00%	0	0.00%
3DS	1	0.12%	1	0.16%	2	0.28%	2	0.38%	0	0.00%	0	0.00%	0	0.00%	0	0.00%
4AL	18	2.10%	15	2.33%	6	0.83%	6	1.13%	14	1.63%	11	1.71%	5	0.69%	4	0.75%
4AS	5	0.58%	5	0.78%	6	0.83%	5	0.94%	0	0.00%	0	0.00%	0	0.00%	0	0.00%
4BL	11	1.28%	10	1.56%	6	0.83%	6	1.13%	3	0.35%	3	0.47%	4	0.55%	4	0.75%
4BS	6	0.70%	5	0.78%	13	1.80%	10	1.89%	4	0.47%	3	0.47%	4	0.55%	3	0.57%
4DL	4	0.47%	4	0.62%	5	0.69%	5	0.94%	0	0.00%	0	0.00%	1	0.14%	1	0.19%
4DS	2	0.23%	2	0.31%	5	0.69%	4	0.75%	0	0.00%	0	0.00%	1	0.14%	1	0.19%
5AL	7	0.81%	5	0.78%	3	0.41%	3	0.57%	3	0.35%	2	0.31%	1	0.14%	1	0.19%
5AS	1	0.12%	1	0.16%	2	0.28%	2	0.38%	1	0.12%	1	0.16%	1	0.14%	1	0.19%
5BL	31	3.61%	28	4.35%	14	1.94%	14	2.64%	12	1.40%	12	1.87%	6	0.83%	6	1.13%
5BS	7	0.81%	5	0.78%	6	0.83%	5	0.94%	2	0.23%	2	0.31%	1	0.14%	1	0.19%
5DL	8	0.93%	7	1.09%	15	2.07%	14	2.64%	2	0.23%	2	0.31%	6	0.83%	6	1.13%
5DS	4	0.47%	3	0.47%	6	0.83%	5	0.94%	0	0.00%	0	0.00%	0	0.00%	0	0.00%
6AL	22	2.56%	17	2.64%	9	1.24%	7	1.32%	6	0.70%	5	0.78%	3	0.41%	3	0.57%
6AS	8	0.93%	8	1.24%	11	1.52%	10	1.89%	5	0.58%	5	0.78%	4	0.55%	4	0.75%
6BL	7	0.81%	6	0.93%	3	0.41%	2	0.38%	4	0.47%	3	0.47%	1	0.14%	1	0.19%
6BS	7	0.81%	5	0.78%	2	0.28%	2	0.38%	5	0.58%	4	0.62%	0	0.00%	0	0.00%
6DL	11	1.28%	10	1.56%	7	0.97%	7	1.32%	3	0.35%	3	0.47%	1	0.14%	1	0.19%
6DS	5	0.58%	3	0.47%	2	0.28%	2	0.38%	4	0.47%	2	0.31%	1	0.14%	1	0.19%
7AL	9	1.05%	8	1.24%	7	0.97%	6	1.13%	6	0.70%	5	0.78%	4	0.55%	4	0.75%
7AS	5	0.58%	5	0.78%	8	1.11%	7	1.32%	0	0.00%	0	0.00%	0	0.00%	0	0.00%
7BL	10	1.16%	10	1.56%	4	0.55%	4	0.75%	5	0.58%	5	0.78%	3	0.41%	3	0.57%
7BS	3	0.35%	3	0.47%	4	0.55%	4	0.75%	4	0.47%	4	0.62%	1	0.14%	1	0.19%
7DL	15	1.75%	10	1.56%	12	1.66%	12	2.26%	5	0.58%	2	0.31%	2	0.28%	2	0.38%
7DS	8	0.93%	4	0.62%	6	0.83%	6	1.13%	1	0.12%	1	0.16%	1	0.14%	1	0.19%
Unmapped	49	5.70%	35	5.44%	63	8.71%	46	8.68%	460	53.55%	358	55.68%	444	61.41%	341	64.34%
Mapped	810	94.30%	608	94.56%	660	91.29%	484	91.32%	399	46.45%	285	44.32%	279	38.59%	189	35.66%

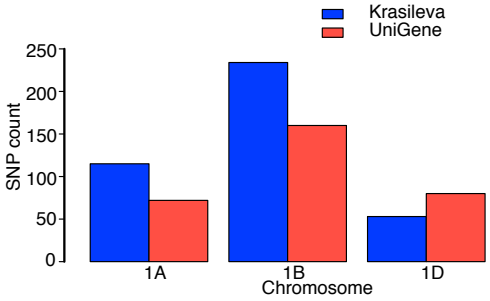


Figure 3.8: Location of SNPs with $BFR > 6$ according to the best alignment of the UniGene (red) and UCW (blue) gene models to the flow-sorted group 1 chromosomes from the CSS (Mayer et al., 2014). Figure adapted from Ramirez-Gonzalez et al. (2015c).

trait. In practice, we only observe clusters of SNPs with enriched BFRs near the centromere of chromosome 1B (Figures 3.9a, 3.10b).

The location of the clusters with an enrichment of SNPs near the centromere is not expected on a random selection of genes, as the gene density increases with the distance to the centromere (Akhunov et al., 2003). This suggests that the experiment was successful on finding SNPs linked to *Yr15*. There are several factor that prevent a clear peak; these include the biases induced by the differential expression and the fragmented reference sequence, with scaffolds that are not long enough to span genetic positions. Since there are several SNPs with a high BFR and the genetic map is not dense enough to locate a single region linked to *Yr15*, multiple criteria were needed to prioritise SNPs that were more likely to yield successful genetic markers.

3.7 Assay selection

Three independent criteria were used to prioritise the SNPs for marker development and validation:

High BFR. SNPs with a $BFR > 6$ in at least two independent bulk replicates or in either of the *in silico* mixes were selected to ensure consistency and recover SNPs with a low coverage on a particular bulk.

Group 1S. SNPs that are in CSS scaffolds in the short arm of chromosome group 1 were selected. The selection included SNPs from the A, B and D genomes because the best hit to each gene model may be missing from the CSS assembly. Therefore, in cases where one or more of the homoeologous genes is missing from the reference, reads might be assigned to the wrong sub-genome. This is

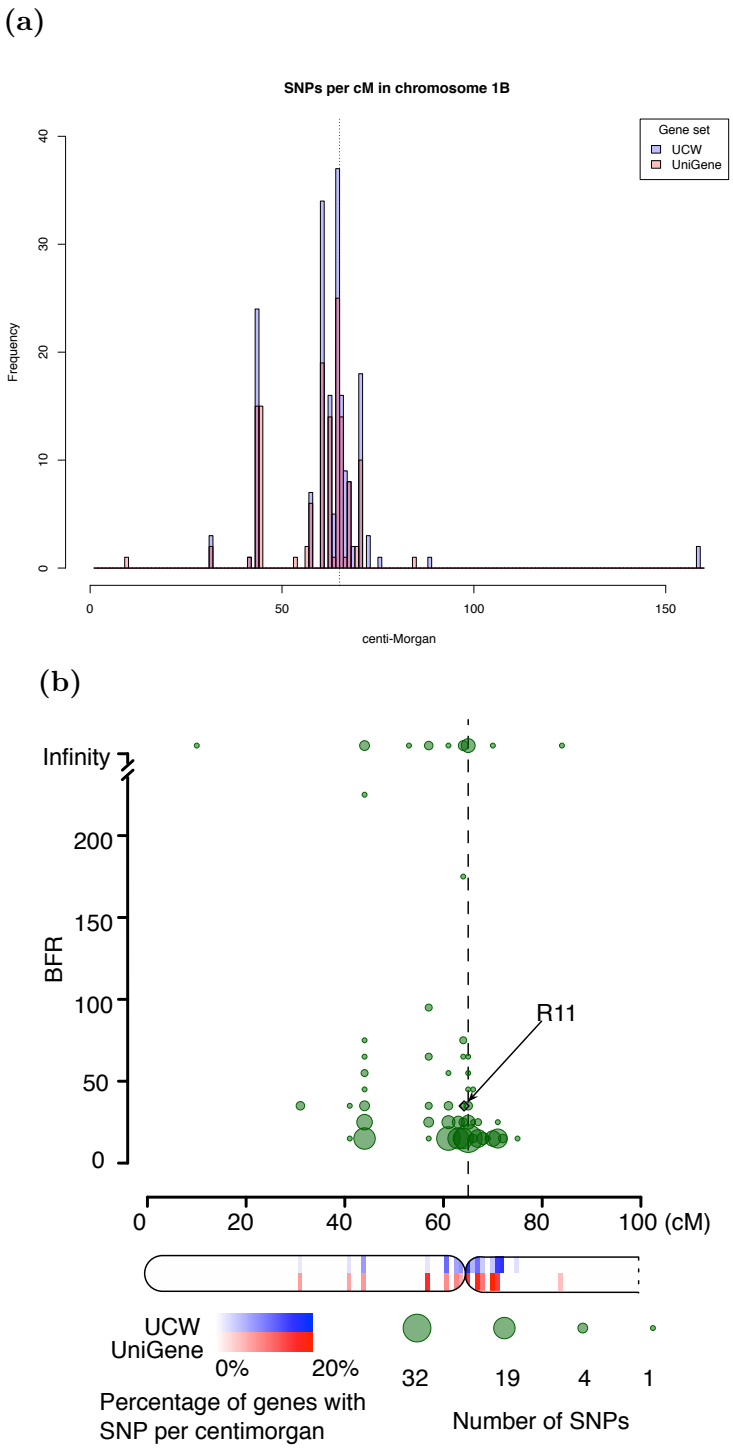


Figure 3.9: *In silico* location of SNPs with $BFR > 6$. (a) Number of SNPs with $BFR > 6$ per cM in chromosome 1B. (b) BFRs of mapped genes with SNPs on chromosome 1B. The area of the circle represents the number of SNPs clustered by location (windows size: 10 cM) and BFR (window size: 5cM). R11 is the only marker near the *Yr15* locus that had a corresponding position in the genetic map. The percentage of genes with SNPs per cM is also illustrated based on UCW (blue) and UniGene (red) gene models. The centromere is imputed by the centre of a window of 10 cM where the short arm switches to the long in the genetic map. BFRs correspond to those from the mixed *in silico* bulk S1 + S2 + S3/R1 + R2 + R3. Adapted from (Ramirez-Gonzalez et al., 2015c).

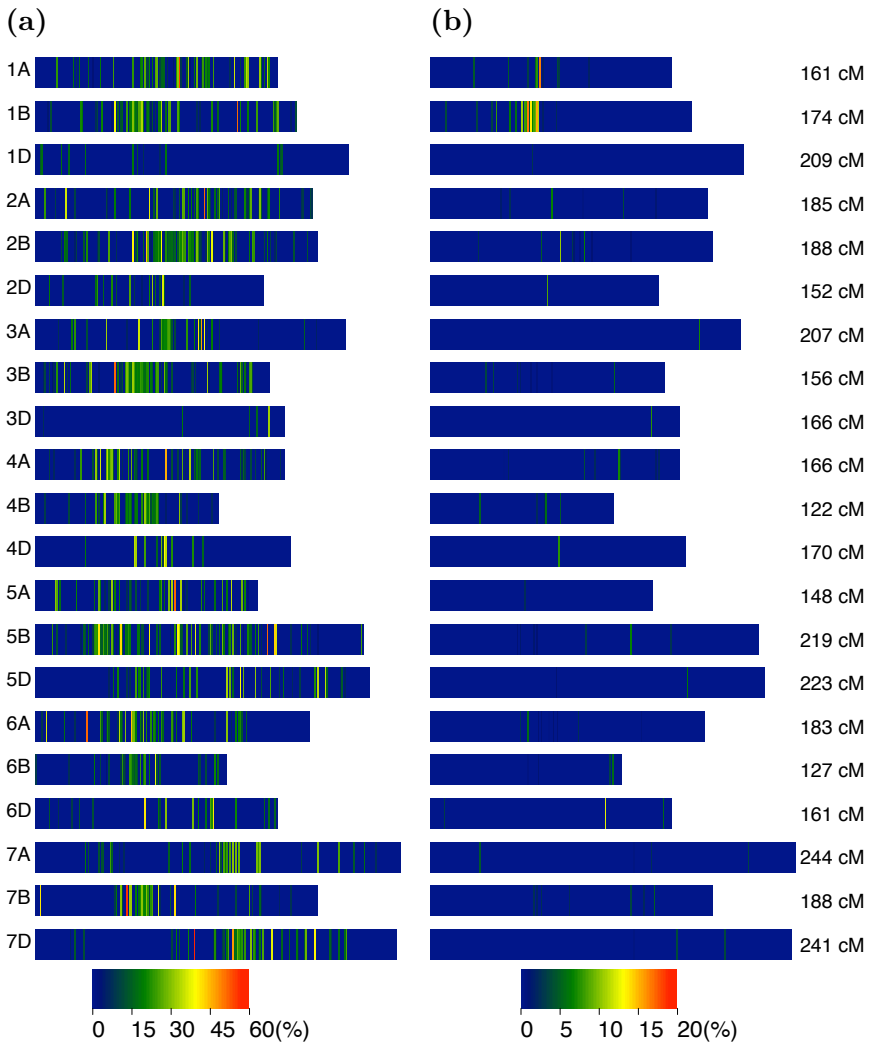


Figure 3.10: Genetic location of genes with SNPs between AVS and Yr15. The colour scale indicates the percentage of genes with SNPs per centi-Morgan (cM) across the 21 wheat chromosomes. The location of the genes was determined by the best alignment to the CSS scaffolds, and the location of these was determined by their position on a genetic map (Wang et al., 2014) (a). All the SNPs between progenitors. Note the lack of enrichment across any individual chromosome. (b) SNPs with BFR>6. Note the enrichment in Chromosome 1B

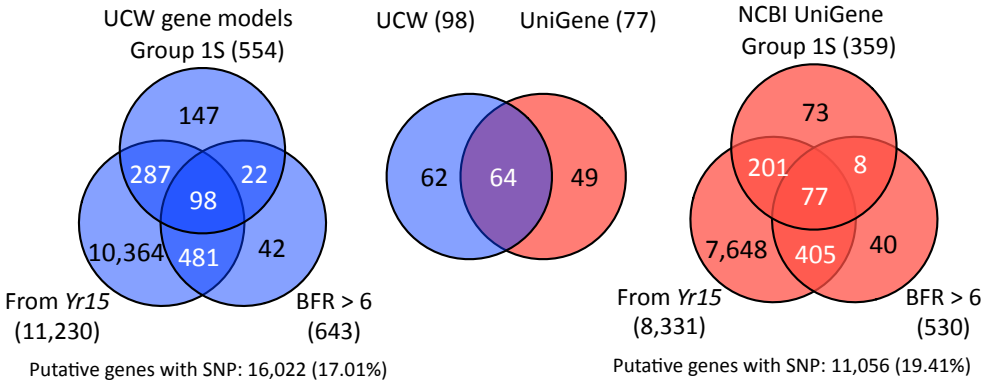


Figure 3.11: Selection criteria for marker design. Venn diagrams based on the three selection criteria (SNP in the short arm of chromosome group 1; SNP has a $BFR > 6$; and SNP is from the *Yr15* parent) for the UCW (blue) and UniGene (red) gene models. The centre diagram shows the intersection between common genes matching all three criteria across both data sets. Note that the numbers are not directly additive as in cases, multiple models from one reference set will relate to a single gene model in the other values. Published in (Ramirez-Gonzalez et al., 2015c)

consistent with the *in silico* genetic map and with previous studies (Murphy et al., 2009; Peng et al., 2000; Sun et al., 1997).

***Yr15* parent.** The SNPs should originate from the *Yr15* parent to ensure that the SNP is coming from the *T. dicoccoides* introgression and not from a SNP in the AVS genetic background, who would be less useful in breeding programs with a different background.

Only SNPs meeting the three criteria were selected for further analysis.

Applying these multiple criteria, the number of genes with a putative SNP went down from over 27,000 to just 175; 77 and 98 from the UniGene and UCW gene sets, respectively. As the two gene sets originate from independent sources, an overlap between the two selected sets is to be expected. When we aligned the 77 and 98 genes from the two collections, we indeed found that around half of the genes overlap (Figure 3.11). The 50 SNPs with the highest BFRs, out of the 175 genes, were selected for validation; fifteen of them were found to be redundant between references, resulting in a final set of 35 SNPs to validate.

The separate bulks and the *in silico* mixes were evaluated in detail to understand the behaviour and value of having multiple bulks. The initial expectation was that the number of SNPs with $BFR = \infty$ should

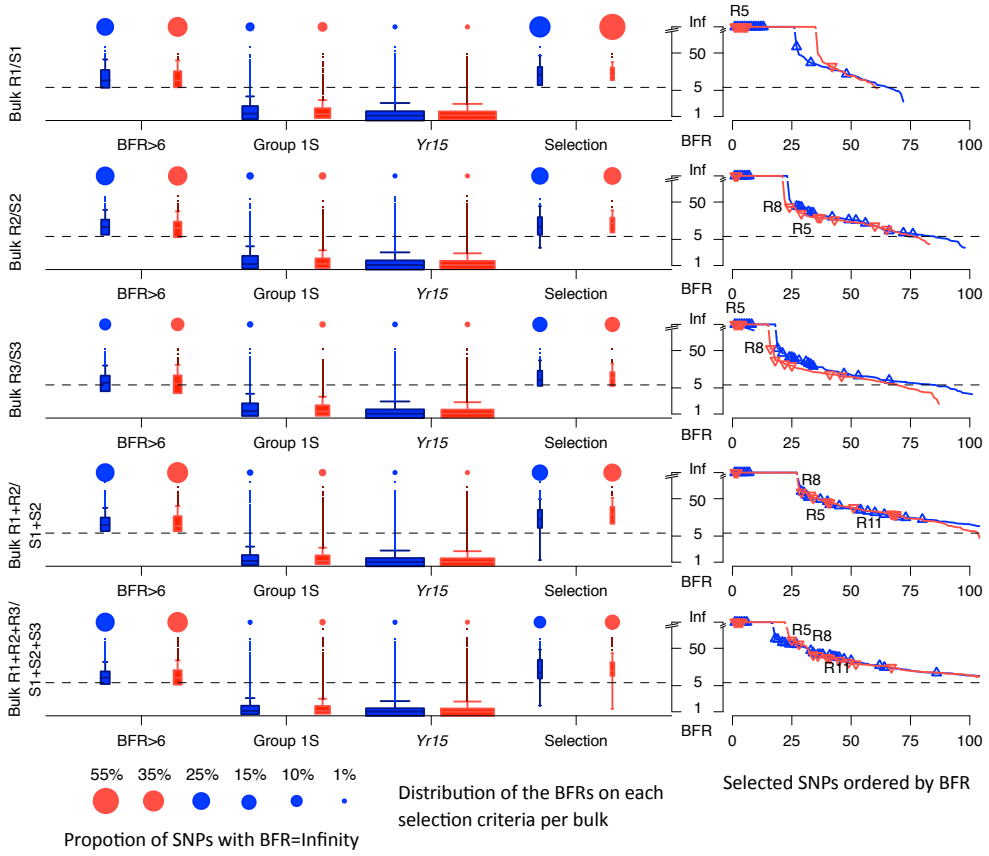


Figure 3.12: BFRs of selected SNPs across the individual bulks and in silico mixes (UCW, red; UniGene, blue). The dotted line represents the BFR threshold of 6 (logarithmic scale). Left: Distribution of the BFRs for each selection criteria and the selected SNPs for validation. The circles on the top of each plot represent the percentage of SNPs with $BFR = \infty$. The Selection may include SNPs with $BFR < 6$ when the same SNP has a higher score on the complementing reference (ie. $BFR > 6$ on UCW, but $BFR < 6$ on UniGenes). Right: The BFR values of selected SNPs were sorted in descending order across the different bulks and according to their origin. Validated SNPs are indicated by open triangles, and SNPs corresponding to markers R5, R8 and R11 are labelled across different bulks and mixes. Note that some SNPs are below the threshold in a specific bulk as they meet the BFR criteria across others.

drop in the mixes, as the improved coverage should reduce the number of instances where the absence of an allele is due to the lack of coverage on a particular sample. However, the opposite happened, the additional coverage in the *in silico* mixes recovered SNPs in genes with a low expression at the time of the sampling (Figure 3.12). Some SNPs were present across all the samples, however the value of the BFR changed depending on the sample (e.g. marker R5). In some cases a SNP was missing in an individual bulk, but present in the rest and also in the mixes (e.g. marker R8). The main parameter affecting the scoring was the coverage in the sample for each particular gene, hence a strategy with a consistent coverage would be preferred for this kind of analysis. Previous studies have shown that a coverage of less than 5x is sufficient to call SNPs in model organisms with a high-quality reference (Schneeberger and Weigel, 2011). However, the results on this study are in line with other studies using populations for SNP calling (Abe et al., 2012; Takagi et al., 2013a). The non-uniform distribution of the coverage in RNA-Seq experiments affects the number of reads that can be used to call for SNPs, especially on genes with a low expression level (Mortazavi et al., 2008).

Around 60% of the gene models, across both references, had a unique hit with greater than 99% sequence identity to a single CSS scaffold (Table 3.8). This is likely because the gene models don't have a unique mapping between gene sets because in cases where only one homoeologue is present in one reference, all the homoeologues in the complementary reference will map to the only represented gene in the original set of genes. To reduce the number of spurious SNPs we used IUPAC ambiguity codes (Section 1.3, Cornish-Bowden (1985)) when two different alleles were observed. This had the side effect that in order to keep only high confidence SNPs we required a higher coverage ($> 20x$). On the original study introducing the BFR in tetraploid wheat, the authors show that increasing the coverage, from $8x$ to $16x$, reduces the putative SNPs by 60%, but the validated SNPs increase from 57% to 83% (Trick et al., 2012). Hence, a compromise between increasing the minimum coverage at the cost of reducing the SNP candidates had to be reached in line with the objectives and available resources for this particular study.

Table 3.8: Number of genes (and SNPs) with a unique hit (> 99% sequence identity) to a single wheat survey sequence scaffold.

Chromosome 1		All SNPs		BFR>6		% BFR>6	
		SNP	Genes	SNP	Genes	SNPs	Genes
UCW	Unique	5,283	1,245	311	214	5.89%	17.19%
	Total	8,086	1,954	486	330	6.01%	16.89%
	Percentage	65.34%	63.72%	63.99%	64.85%		
UniGene	Unique	3,687	745	213	139	5.78%	18.66%
	Total	6,422	1,318	386	246	6.01%	18.66%
	Percentage	57.41%	56.53%	49.17%	56.07%		
UCW	Unique	8,970	1,990	524	353	5.84%	17.74%
+	Total	14,508	3,272	872	576	6.01%	17.60%
UniGene	Percentage	61.83%	60.82%	60.09%	61.28%		

All SNPs		All SNPs		BFR>6		% BFR>6	
		SNP	Genes	SNP	Genes	SNPs	Genes
UCW	Unique	39,247	9,585	481	368	1.23%	3.84%
	Total	66,426	16,022	859	643	1.29%	4.01%
	Percentage	59.08%	59.82%	56.00%	57.23%		
UniGene	Unique	27,292	5,698	344	252	1.26%	4.42%
	Total	52,262	11,056	723	530	1.38%	4.79%
	Percentage	52.22%	51.54%	47.58%	47.55%		
UCW	Unique	66,539	15,283	825	620	1.24%	4.06%
+	Total	118,688	27,078	1,582	1,173	1.33%	4.33%
UniGene	Percentage	56.06%	56.44%	52.15%	52.86%		

3.8 SNP Validation

KASP assays were designed to validate and generate a genetic map of the *Yr15* locus for the 35 selected SNPs. To automate the design of genome-specific primers for polyploid organisms PolyMarker was developed (Chapter 2). Out of the 35 assays to design, 17 were designed as specific, 9 as semi-specific to chromosome 1BS, and 9 were not specific because there was no information for the homoeologues on the CSS scaffolds. PolyMarker also identified putative homoeologous variants (between genomes, as opposed to between varieties) that were in the list of candidate SNPs, but were not identified previously (Figure 2.11; Table 3.9).

To validate if the 35 SNPs were polymorphic across the parents and diagnostic for *Yr15* we tested them in the progenitors plus six commercial varieties, three containing *Yr15* (Ochre, Boston and, Cortez) and three without it (Shamrock, Robigus and, Cadenza). Two of the lines without *Yr15* have *T. dicoccoides* in their pedigree (Shamrock and Robigus), as it is the donor species of *Yr15* (McIntosh et al., 1995). This test panel allows to test if the SNPs are only diagnostic to *T. dicoccoides* instead of *Yr15*. On the test panel, 28 (80%) SNPs were polymorphic across the parents and three of them where diagnostic to *Yr15* (R5, R8, R33). From the five homoeologous SNPs, three of them were monomorphic and

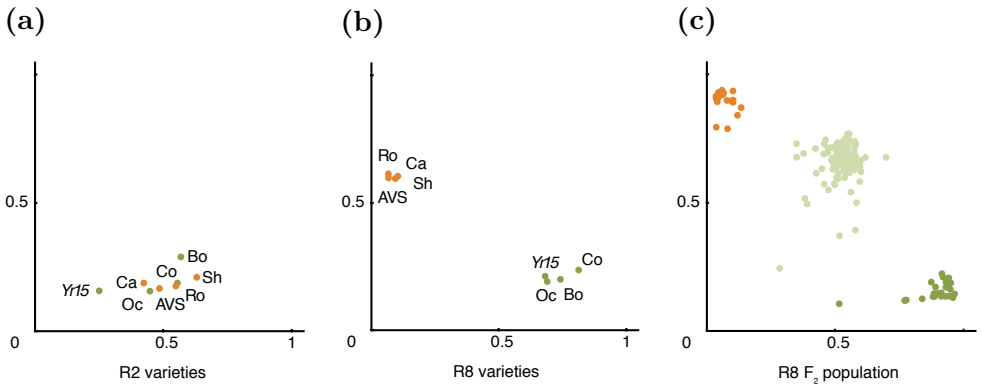


Figure 3.13: KASP output from the wheat variety panel with (Ochre, Boston, Cortez) and without (Robigus, Cadenza and Shamrock) *Yr15*. Marker R2 (a) is monomorphic while R8 (b) is polymorphic between varieties known to carry the gene. Marker R8 results for the F2 population (c) showing three distinct clusters. The central cluster (light green) is comprised of heterozygous individuals, whereas clusters near the axes are homozygous for either AVS (VIC; orange) or *Yr15* (FAM; dark green).

two polymorphic, suggesting that PolyMarker is effective on detecting which assays are less likely to work (Table 3.10; Figure 3.13a,b). The segregation of the SNPs in the full F_2 population (Section 3.2, Figure 3.13c) and a genetic map was produced (Section 3.9).

3.9 Genetic map

Initially, the 28 polymorphic markers were used to genotype a subset of 66 plants from the F_2 population. From those, 23 (82%) were linked to *Yr15* and several markers map in a small interval around *Yr15* (Figure 3.14a; Table 3.10), confirming that the multiple-criteria strategy(Section 3.7) for selecting candidate SNPs was effective. Then, the complete F_2 population was assessed with:

- the seven markers that were most closely linked to *Yr15*, including two of the diagnostic markers from the variety panel (R5 and R8),
- The flanking SSR microsatellite markers used by UK breeders for germoplasm selection (*Xbarc8* and *Xgwm413*). These correspond to the best markers available to breeders at the time of the study.
- A marker based on barley-wheat synteny (R43) which met the selection criteria, but was not on the original set of 50 markers with high BFR.

The F_2 population consisted on 232 plants with phenotypic information, of those 196 where genotyped reliably (no more than one data point missing). Using the eight SNP markers and 2 SRRs, the *Yr15* locus was mapped to an interval of 0.77cM, with R8/xgwm413 0.26cM distal, and R5/R11 0.77cM proximal from *Yr15* (Figure 3.14b,c).

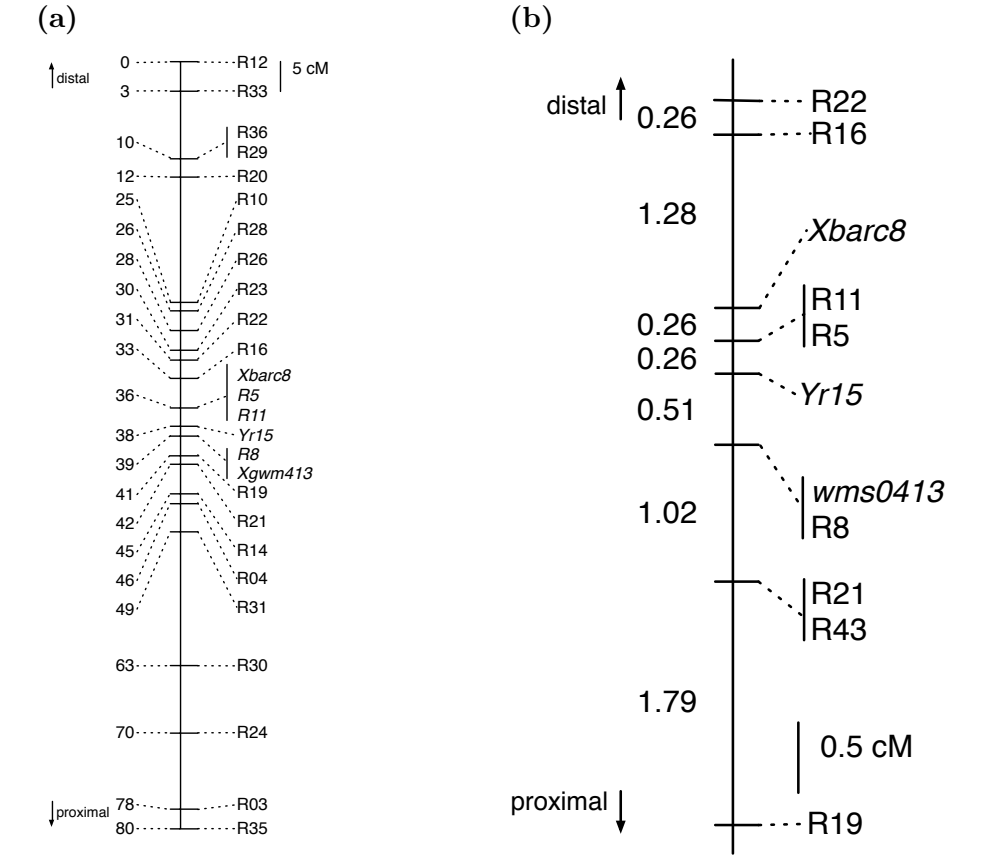
The sub-cM resolution is expected in a F_2 population of 196 individuals, as 392 gametes should provide n average resolution of 0.26cM. Despite the fact that none of the selected markers have perfect linkage to *Yr15*, the resulting genetic map is an improvement in the resolution of the map for the locus and it enables the shift to SNP markers from microsatellites. The former has become the preferred marker system in Marker Assisted Selection (MAS) pipelines in breeding programmes.

Table 3.9: Primer details for the markers to validate.

Assay ID	Polymorphism_type	AVS-specific primer	Yr15-specific primer	Common primer	Specificity	Orientation
R1	non-homeologous	aactggtaatggtgcagCgG	aactggtaatggtgcagCgC	ttcaggataacacAggagatgtT	chromosome_semispecific	reverse
R2	non-homeologous	acatcaattcttcaggaaagctctaC	acatcaattcttcaggaaagctctaT	gcacagcttctcgtgttcTT	chromosome_specific	forward
R3	non-homeologous	acgtggagaacctagattgcG	acgtggagaacctagattgcC	ccttttaggtgcgccaactT	chromosome_semispecific	reverse
R4	non-homeologous	agactctttgggcagtggatC	agactctttgggcagtggatT	cctcgggcgatctattctcT	chromosome_specific	forward
R5	non-homeologous	agtcaacttggattacactgaagtT	agtcaacttggattacactgaagtC	agatatcacactgaacatactgatgaG	chromosome_specific	reverse
R6	non-homeologous	caagatgaagatgaagaggaatatgaT	caagatgaagatgaagaggaatatgaC	gCttgaccctgtaatcatactcG	chromosome_semispecific	forward
R7	non-homeologous	caccaccaTggaggccaC	caccaccaTggaggccaT	cgccgtggtagtgtccgG	chromosome_specific	forward
R8	non-homeologous	cagatccccggttctctcaaG	cagatccccggttctctcaaA	cccccaaatgatcgagaata	chromosome_inspecific	reverse
R9	non-homeologous	caggtgctgaaatgcatcC	caggtgctgaaatgcatcT	cggcctatcttcaggtctgt	chromosome_inspecific	reverse
R10	non-homeologous	cattcgtcgcgccttctacG	cattcgtcgcgccttctacA	tcctaactcatatgcatgactcAC	chromosome_specific	reverse
R11	non-homeologous	ccattctgatcaaggtcactgtcG	ccattctgatcaaggtcactgtcA	ttctgtaTggcaaCgggagC	chromosome_specific	reverse
R12	homeologous	cttagccagtgaaccAggcC	cttagccagtgaaccAggcT	ggctgtttgttacCgtggaG	chromosome_specific	reverse
R14	non-homeologous	gacTacAggtgcgatcccC	gacTacAggtgcgatcccT	ctcgcctgccagtcgTaT	chromosome_specific	forward
R15	homeologous	gactagggctaccAttgttgA	gactagggctaccAttgttgC	agccctgCtaacaatggcaA	chromosome_specific	reverse
R16	non-homeologous	gatgtaagcTAtgactggCgC	gatgtaagcTAtgactggCgT	tgcaactgatctttagcaggC	chromosome_semispecific	reverse
R17	non-homeologous	gcaAcaacaaCaaCaagtggT	gcaAcaacaaCaaCaagtggC	cctcaacctgcttgttgttgT	chromosome_specific	forward
R19	non-homeologous	gcctgatttttaattcgctccaG	gcctgatttttaattcgctccaA	agagcactgatgatgacccC	chromosome_specific	reverse
R20	non-homeologous	gctgtatcctcttgaaaaaggcT	gctgtatcctcttgaaaaaggcC	ttaggcatgtcagaaatgtagaaaa	chromosome_semispecific	forward
R21	non-homeologous	gcttcaaacatgccggctG	gcttcaaacatgccggctT	cggtctttttcaaccagggC	chromosome_semispecific	forward
R22	homeologous	gctTgtCttaaagccAtttccA	gctTgtCttaaagccAtttccG	gcctatcgttCgctaaactctaacT	chromosome_specific	reverse
R23	non-homeologous	gctttaggcactatggattcAcC	gctttaggcactatggattcAcT	caggtttctgttcgacctcA	chromosome_specific	forward
R24	non-homeologous	ggaggtcctacacgcgtctT	ggaggtcctacacgcgtctG	ctccaaaagaggggcatcattT	chromosome_semispecific	forward
R25	non-homeologous	gggttcctcacctgcgcC	gggttcctcacctgcgcT	ctctTtgcaatcggccagc	chromosome_inspecific	reverse
R26	non-homeologous	gtCttcgcCggcacCacC	gtCttcgcCggcacCacT	agtggatcttgccgatctcg	chromosome_inspecific	forward
R28	non-homeologous	tagatgagaccttggaCggA	tagatgagaccttggaCggG	cagtcatctaatgcggaacattcA	chromosome_semispecific	reverse
R29	non-homeologous	TatggtGtggccTtccccG	TatggtGtggccTtccccA	cgagctcgctgatgaacttG	chromosome_specific	forward
R30	non-homeologous	tcagcagccctttttaacccaA	tcagcagccctttttaacccaT	agtaaatcgggcacggttgt	chromosome_inspecific	reverse
R31	homeologous	tcatccatgtatatGaaTccaagcC	tcatccatgtatatGaaTccaagcA	tcacgcctgcaacAttcaaaT	chromosome_specific	reverse
R32	homeologous	tccaatcttatggctttgcttctG	tccaatcttatggctttgcttctT	caggtgatgtagatgctgagaC	chromosome_semispecific	reverse
R33	non-homeologous	tccttcctgctatagctgaaagG	tccttcctgctatagctgaaagT	ccctttgcctgccatgtaga	chromosome_inspecific	forward
R34	non-homeologous	tctgagatgatgatactTtgtggG	tctgagatgatgatactTtgtggA	actggggatgccctctgtat	chromosome_inspecific	forward
R35	non-homeologous	tgaaagagtggaatttcttgttgT	tgaaagagtggaatttcttgttgC	ctttTagctgcttaattctattgcttC	chromosome_specific	forward
R36	non-homeologous	tgaaatgccttgtcaatgccA	tgaaatgccttgtcaatgccG	ATGCGAATTGGGGAATTAAA	chromosome_inspecific	reverse
R37	non-homeologous	tgcatatgcctgaagagactcG	tgcatatgcctgaagagactcA	tgtccacctactcaagtctgc	chromosome_inspecific	reverse
R38	non-homeologous	tgGcCaagTtTttctgcaagaT	tgGcCaagTtTttctgcaagaG	tgtaggaaggaactcCgaagtA	chromosome_specific	forward
R40	non-homeologous	tgcatatgcctgaagagactcA	tgcatatgcctgaagagactcG	agtccgctaaagcattgcct	chromosome_nonspecific	reverse
R43	non-homeologous	tcgctgatttcatcatgtcccA	tcgctgatttcatcatgtcccG	tcaggtgctgcaaatttgagG	chromosome_semispecific	forward

Table 3.10: Results of validation of primers on the progenitors (AVS and *Yr15*, varieties known to contain *Yr15* (Cortez, Ochre and, Boston) and, varieties without *Yr15* (Robigus, Cadenza and, Shamrock). Shamrock and Robigus have *T. dicoccoides* introgressions. The bold markers are diagnostic in the panel (R5, R8, R88) or in the genetic map (R11).

Assay ID	Gene set	Gene model name	SNP	Yr15	Ochre	Boston	Cortez	Shamrock	Robigus	Cadenza	AVS	Polymorphic	Linked *Yr15*	comment
				Yr15+				*Yr15-*						
R1	UCW	UCW_Tt-k55_contig_8830;tt-k21_contig_10204	C341G	A	H	A	A	A	-	A	B	Yes	*	segregation distortion
R2	UniGene v60	gnl|UG|Ta#S13126619	C491T	B	B	B	B	B	B	B	B	No	-	
R3	UCW	contig95240	C220G	H	B	B	B	B	B	B	B	Yes	Yes	
R4	UCW	contig105384	C1227T	A	B	B	B	B	B	B	B	Yes	Yes	
R5	UniGene v60	gnl|UG|Ta#S58861868	A214G	**A**	**A**	**A**	**A**	**B**	**B**	**B**	**B**	Yes	Yes	
R6	UCW	KukriC706_1	T2979C	A	H	B	B	B	B	H	H	Yes	No	
R7	UniGene v60	gnl|UG|Ta#S37932863	C281T	H	A	A	A	B	B	A	B	Yes	No	
R8	UniGene v60	gnl|UG|Ta#S58863387	T241C	**B**	**B**	**B**	**B**	**A**	**A**	**A**	**A**	Yes	Yes	
R9	UniGene v60	gnl|UG|Ta#S58892239	C303T	H	B	A	B	B	B	H	B	Yes	No	
R10	UCW	UCW_Tt-k63_contig_79829	C207T	H	A	B	A	B	B	B	B	Yes	Yes	
R11	UCW	UCW_Tt-k45_contig_39011	C726T	**A**	**A**	**A**	**H**	**-**	**B**	**B**	**B**	Yes	Yes	
R12	UCW	contig50308	G587A	-	H	H	H	B	B	A	B	Yes	Yes	
R14	UniGene v60	gnl|UG|Ta#S44692929	C549T	A	A	-	A	A	B	-	B	Yes	Yes	
R15	UCW	UCW_Tt-k51_contig_2344;tt-k55_contig_2091	T686G	A	A	A	A	A	A	A	A	No	-	
R16	UniGene v60	gnl|UG|Ta#S17898149	G227A	A	A	B	A	B	B	B	B	Yes	Yes	
R17	UCW	CL3339Contig1	T509C	H	H	H	H	H	H	H	H	No	-	
R19	UCW	UCW_Tt-k21_contig_8407;tt-k61_contig_5972	C1405T	A	B	B	B	B	B	B	B	Yes	Yes	
R20	UCW	UCW_Tt-k21_contig_8407;tt-k61_contig_5972	T1102C	A	B	B	B	-	B	B	B	Yes	Yes	
R21	UCW	UCW_Tt-k31_contig_53804;tt-k41_contig_31582	G1810T	H	B	B	B	B	B	B	B	Yes	Yes	
R22	UCW	UCW_Tt-k31_contig_14966	T408C	A	A	A	A	A	A	A	B	Yes	Yes	
R23	UCW	UCW_Tt-k51_contig_12731;tt-k55_contig_13077;tt-k61_contig_18734	C50T	A	H	H	H	H	-	H	B	Yes	Yes	
R24	UCW	UCW_Tt-k55_contig_8830;tt-k21_contig_10204	T3005G	H	H	B	H	B	B	B	B	Yes	Yes	
R25	UCW	UCW_Tt-k63_contig_79829	G184A	A	A	A	A	H	H	A	A	No	-	
R26	UCW	UCW_Tt-k21_contig_3794	C702T	H	A	B	A	B	B	B	B	Yes	Yes	
R28	UCW	KukriC3701_1	T1053C	A	A	B	A	B	B	B	B	Yes	Yes	
R29	UCW	UCW_Tt-k55_contig_8640;tt-k41_contig_8875	G783A	H	A	B	A	B	B	B	B	Yes	Yes	
R30	UCW	UCW_Tt-k55_contig_8830;tt-k21_contig_10204	T2184A	A	A	B	A	B	B	A	B	Yes	Yes	
R31	UCW	UCW_Tt-k45_contig_22098	G683T	A	B	A	B	B	B	A	B	Yes	Yes	
R32	UCW	UCW_Tt-k21_contig_33188;tt-k25_contig_30647	C596A	H	A	A	A	A	A	A	H	No	-	
R33	UniGene v60	gnl|UG|Ta#S58861868	G486T	**A**	**A**	**A**	**A**	**B**	**B**	**B**	**B**	Yes	Yes	
R34	UCW	UCW_Tt-k31_contig_34099	G1713A	H	A	B	-	B	B	B	B	Yes	No	
R35	UniGene v60	gnl|UG|Ta#S58900202	T889C	A	B	B	B	B	B	B	B	Yes	Yes	
R36	UCW	UCW_Tt-k55_contig_8830;tt-k21_contig_10204	T2349C	H	H	H	H	H	-	H	B	Yes	Yes	
R37	UCW	UCW_Tt-k31_contig_34099	C846T	B	B	B	B	B	B	B	B	No	-	
R38	UniGene v60	gnl|UG|Ta#S58840501	T179G	B	B	B	B	B	B	B	B	No	-	
R40	UCW	UCW_Tt-k31_contig_34099	C846T	A	H	B	A	-	B	B	B	Yes	No	based on barley synteny
R43	UniGene v60	gnl|UG|Ta#S58843705	G268A	A	B	B	-	-	B	-	B	Yes	Yes	based on barley synteny



(c)

R22	R16	*Xbarc8*	R11	R5	Yr Score	R8	*Xgwm413*	R43	R21	R19	Individuals
B	B	B	B	B	R	B	B	B	B	B	51
B	B	H	H	H	R	H	H	H	H	H	1
B	B	B	B	B	R	B	B	B	B	H	6
H	H	B	B	B	R	B	B	B	B	H	1
H	H	B	B	B	R	B	B	B	B	B	1
H	H	H	B	B	R	B	B	B	B	B	1
H	H	H	H	H	R	H	H	B	B	-	1
H	H	H	H	H	R	H	H	H	H	H	88
A	A	A	A	A	S	A	A	A	A	A	37
H	H	H	H	H	S	A	A	-	A	A	1
H	H	H	H	H	R	H	H	A	A	A	2
A	H	H	H	H	R	H	H	H	H	H	1
A	A	H	H	H	R	H	H	H	H	H	2
A	A	A	A	A	R	H	H	H	H	H	1
A	A	A	A	A	S	H	H	H	H	H	1
A	A	A	A	A	S	A	A	H	H	H	1

Figure 3.14: Genetic maps for *Yr15*. (a) Genetic map of the test panel from 50 individuals. (b) Genetic map from 196 individuals from the full population only with the 8 markers previously identified as closer to the *Yr15* locus. (c)Graphical genotype of the 196 F_2 individuals used to develop the genetic map. The alleles are abbreviated according to their origin: A: AVS; B: *Yr15* and H: Heterozygous. Missing calls are indicated by a hyphen.

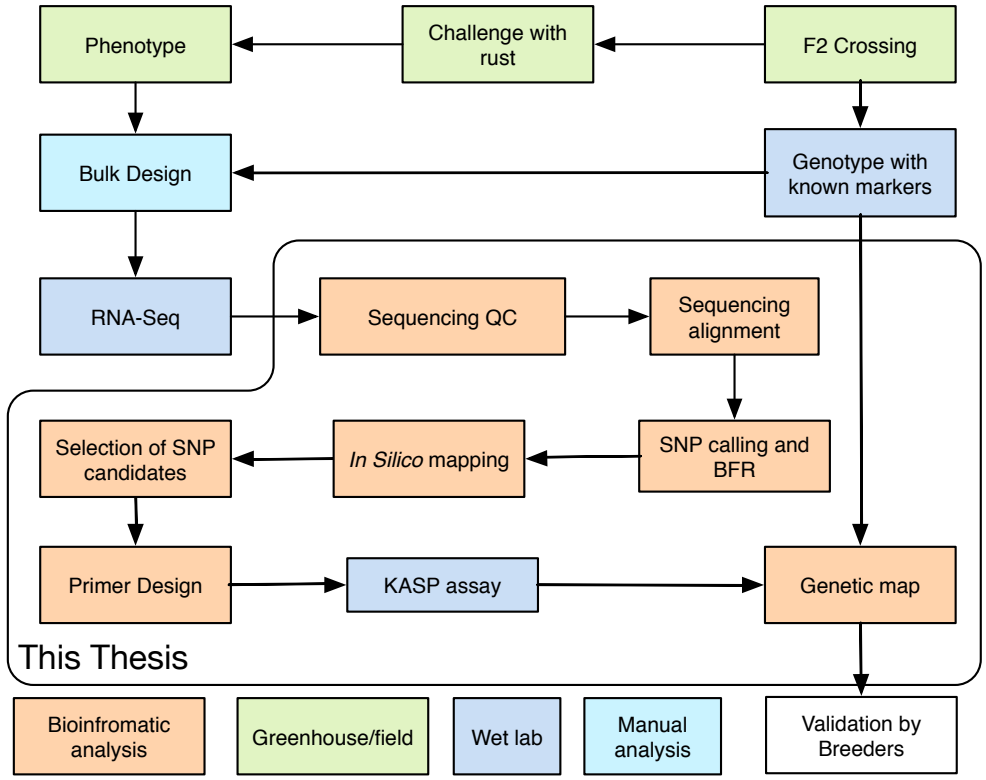


Figure 3.15: Steps used to go from the F_2 population to the genetic map.

3.10 Methods

The data analysis for this PhD required the use of some standard tools and custom developed code. All the code produced for this project is available and updated on the a github repository: `https://github.com/TGAC/bioruby-polyploid-tools`. For clarity, the snippets of code on this section had been simplified by removing the exception handling, type checks and caching mechanism.

3.10.1 Base-call and Quality Control of sequencing reads

The raw output from the Illumina HiSeq 2000 was processed with Casava v1.8 (Illumina, 2011). Lanes 1 and 2, containing multiplexed bulks (Table 3.1) was de-multiplexed with a tolerance of 1 mismatch in the barcode. Lanes 3 and 4 contained the parental sequences without a barcode. The FastQ files where left compressed and in chunks of 40,000, as the default for the BCL conversion pipeline from Casava to allow parallel processing

in a cluster environment. The quality of the sequencing lanes was assessed with FastQC v0.10.1 (Babraham Bioinformatics, 2012).

3.10.2 Alignment reads to gene models

The RNA-Seq reads were aligned with BWA 0.5.9 (Li and Durbin, 2009) to the wheat UniGene database v60 (Pontius et al., 2002) and to the UCW gene models (Krasileva et al., 2013), including the *T. turgidum* and complementary ORFs (MAS Wheat, 2013). The alignments where sorted and stored as single BAM files to have random access (Li et al., 2009).

3.10.3 Bulk Frequency Ratios and SNP calling

To avoid the creation of several temporary files with the coverage information on all the bases I developed a `Ruby` pipeline based on the `bio-samtools` library (Ramirez-Gonzalez et al., 2012), and some of the improvements to work with pileups were published as a follow-up on the library (Etherington et al., 2015). To call for the consensus, the function `Bio::DB::Sam::mpileup` is called to generate the pileup of each gene. As the pileups are used several times during the analysis, a function that caches the current pileup is implemented. The consensus is called by counting how many times each base appears, and if the number of bases is higher than `minumum_ratio_for_iuap_consensus` the base is added to the set of possible bases (Cornish-Bowden, 1985) If there is no coverage at a certain position, the reference base is used, and set as lowercase. If the set of called bases is not empty, the ambiguity code for the observed bases is called, and set as upper case (Listing 3.1). The minimum ratio was done on 0.2 (20%), as it allows calling for a consensus even when more than one homoeologue is mapping to the same reference.

Listing 3.1: Method to call for the consensus on progenitors from a pileup

```
def consensus_iuap(minumum_ratio_for_iuap_consensus)
  minumum_ratio_for_iup_consensus
  @consensus_iuap = self.ref_base.downcase
  bases = self.bases
  tmp = String.new
  bases.each do |k,v|
    if v/self.coverage > minumum_ratio_for_iup_consensus
      tmp << k[0].to_s
    end
    if tmp.length > 0
      @consensus_iuap = Bio::NucleicAcid.to_IUAPC(tmp)
    end
  end
  @consensus_iuap.upcase
end
```

Then, to calculate the BFRs as shown on Figure 3.2 extra extensions for the `Bio::DB::Pileup` were added to get the actual number of bases in the pile (to exclude short insertions and deletions; Listing 3.2), and to calculate the SNP-Index (Listing 3.3).

Listing 3.2: `base_coverage` gets the number of bases called from a single pileup.

```
def base_coverage
  total = 0
  @bases.each do |k,v|
    total += v
  end
  total
end
```

Listing 3.3: `base_ratios` gets the SNP-Index on a single pileup.

```
1 def base_ratios
2   return @base_ratios if @base_ratios
3   bases = self.bases
4   @base_ratios = Hash.new
5   bases.each do |k,v|
6     @base_ratios[k] = v.to_f/self.base_coverage.to_f
7   end
8   @base_ratios
9  end
```

To calculate BFRs the class `Bio::BFRTools::Container` was implemented to contain all the `BIO:DB:Sam` objects corresponding to the progenitors and the bulks. The class `Bio::BFRTools::BFRRegion` was implemented to contain the ratios and consensus sequences of each region. The method `bfr` uses the calculated SNP-Indices on every position, from the point of view of both progenitors (lines 15-16: Listing 3.4, and in the case of lack of coverage the value is set to 0 or `Infinity` (lines 8-13), depending on the progenitor where the base is not called at all. Using this design where the values of each region are calculated at once reduces the number of times the pileup needs to be generated for each sample and allows to have in a single place in memory all the elements to calculate the BFRs, without having to write any temporary files on disc. Also, the fact that the calculation of each region is independent from that for other regions, it is possible to use a computing cluster to distribute the analysis on several nodes.

The code produces a table with the SNP-Indices and BFRs for all the SNPs found in the progenitors. The program was used to calculate the BFRs on the independent conditions (Bulk 1: S1-R1, Bulk 2: S2-R2 and Bulk 3: S3-R3); the *in silico* mixes of bulks 1 and 2; and bulks 1, 2 and 3.

Listing 3.4: Section of the code that

```
1 for i in (0..self.size-1)
2   ratios_1 = @ratios_bulk_1[i]
3   ratios_2 = @ratios_bulk_2[i]
4   BASES.each do |base|
5     if ratios_1[base] == 0 and ratios_2[base] == 0
6       bfr1 = 0
7       bfr2 = 0
8     elsif ratios_1[base] == 0
9       bfr1 = 0
10      bfr2 = Float::INFINITY
11    elsif ratios_2[base] == 0
12      bfr1 = Float::INFINITY
13      bfr2 = 0
14    else
15      bfr1 = ratios_1[base] / ratios_2[base]
16      bfr2 = ratios_2[base] / ratios_1[base]
17    end
18    @BFRs[:first][base] << bfr1
19    @BFRs[:second][base] << bfr2
20  end
21 end
```

3.10.4 *In Silico* mapping

To find the chromosomal position of the SNPs with a high BFR the sequences of the markers with a genetic position from Wang et al. (2014) were aligned with BLAT (Kent, 2002) to the CSS scaffolds (Mayer et al., 2014). The best hit for each query was found and cached using a `Ruby script`. Briefly, the class `Bio::Blat::Report` from `BioRuby` (Goto et al., 2010) was extended to include an iterator only for the best alignment of each query: First, the whole file is iterated (line 5); the alignment with the best score is stored in a hash (lines 7-9) and finally the hash is iterated (line 11). The script found 46,977 scaffolds that contained at least one marker from the map.

Listing 3.5: Extension to `Bio::Blat::Report` that selects the best alignment from a `psl` file from BLAT

```
def self.each_best_hit(text = '')
  emptyHit = Bio::Blat::Report::Hit.new
  emptyHit.score = 0
  best_aln = Hash.new(emptyHit)
  self.each_hit(text) do |hit|
    current_score = hit.score
    if current_score > best_aln[current_name].score
      best_aln[current_name] = hit
    end
  end
  best_aln.each_value { |val| yield  val }
end
```

Then, the UniGenes and the UCW gene models were also aligned with BLAT to the scaffolds that were located in the genetic map. The class `Bio::Blat::Report::Hit` was extended to calculate how many bases are covered in the alignment and the percentage of covered bases in both, the target and query sequences (Listing 3.6). Only the genes that align over 60% of covered bases with an identity of at least 90% were considered. This removes spurious mappings from repetitive regions, while retaining assignment to a homoeologue in the case in which the correct scaffold is not in the genetic map. The genes were also aligned to the full CSS reference, to be able to allocate the genes to a chromosome arm, even when it is not possible to assign a position in neither the genetic map nor to the cDNAs of *Hordeum vulgare* (Mayer et al., 2011) (as deposited in Ensembl! Plants, release 16 (Kersey et al., 2012)). The genetic position of the contigs was used to calculate the density of SNPs between AVS and *Yr15* in the genetic bins for Figure 3.10. This information was used to select the SNPs with high BFR to validate.

Listing 3.6: Extension to `Bio::Blat::Report::Hit` for filtering of spurious alignments.

```
class Bio::Blat::Report::Hit
  def covered
    match + mismatch
  end
  def query_percentage_covered
    covered * 100.0 / query_len.to_f
  end
  def target_percentage_covered
    covered * 100.0 / target_len.to_f
  end
end
```

3.10.5 Primer design and KASP assays

The primer designs for KASP were designed with PolyMarker as described in Chapter 2. The only difference with default settings is that instead of using a template sequence, the sequence for each allele is calculated from the consensus of the alignments. As described in Ramirez-Gonzalez et al. (2015c),

> [the primers] were ordered from Sigma-Aldrich (Gillingham, UK), with primers carrying standard FAM or HEX compatible tails (FAM tail: 5' GAAGGTGACCAAGTTCATGCT 3'; HEX tail: 5' GAAGGTCGGAGTCAACGGATT 3') and the target SNP at the 30 end. Primer mix was set up as recommended by LGC [46 μL dH_2O, 30 μL common primer (100 μM) and 12 μL of each tailed primer (100 μM)] (LGC Genomics, 2014) Assays were tested in 384-well format and set up as 4-μL reactions [2-μL template (10–20 ng of DNA), 1.944 μL of V4 2 $\times$ Kaspar mix and 0.056 μL primer mix]. PCR cycling was performed on a Eppendorf Mastercycler pro 384 using the following protocol: hot-start at 95°C for 15 min, followed by ten touchdown cycles (95°C for 20s; touchdown 65°C, -1°C per cycle, 25 s) then followed by 30 cycles of amplification (95°C 10s; 57°C 60s). As KASP amplicons are smaller than 120 bp, an extension step is unnecessary in the PCR protocol. 384-well optically

> clear plates (Cat. No. E10423000; Starlab Milton Keynes, UK) were read on a Tecan Safire plate reader. Fluorescence was detected at ambient temperature. If the signature genotyping clusters had not formed after the initial amplification, additional amplification cycles (usually 5–10) were conducted, and the samples were read again. Data analysis was performed manually using Klustercaller software (version 2.22.0.5; LGC Hoddesdon, UK).

3.10.6 Genetic map

As described in Ramirez-Gonzalez et al. (2015c):

> JoinMap version 3 (van Ooijen and Voorrips, 2002) was used for linkage analysis and genetic map construction, using default settings. The linkage to *Yr15* was determined using a divergent log-of-odds (LOD) threshold of 3.0, and genetic distances were computed based on recombination frequency.

3.11 Discussion

Re-sequencing the $\sim$ 17Gbp genome of hexaploid wheat is costly and approaches to reduce the required sequenced volume to effectively call for SNPs had been evolving since the conception of this project. Both the RNA and DNA extraction and the sequencing for this project were carried out before the beginning of my PhD (before October 2012). At that time, exome capture was already an established technique for genotyping humans (Ng et al., 2009), however the first exome capture on wheat had just been published, with probes coming from unassembled 454 reads (Winfield et al., 2012); the first probe designed directly from transcripts (Henry et al., 2014) was not published until after the analysis of this section was completed and validated (Figure 3.16). An even more targeted capture for resistance genes (RenSeq), by capturing genes with the NBS-LRR motif, was published while this study was executed (Jupe et al., 2013). On the other hand, RNA-Seq had already been tested for Bulk Segregant Analysis on tetraploid wheat (Trick et al., 2012). Hence,

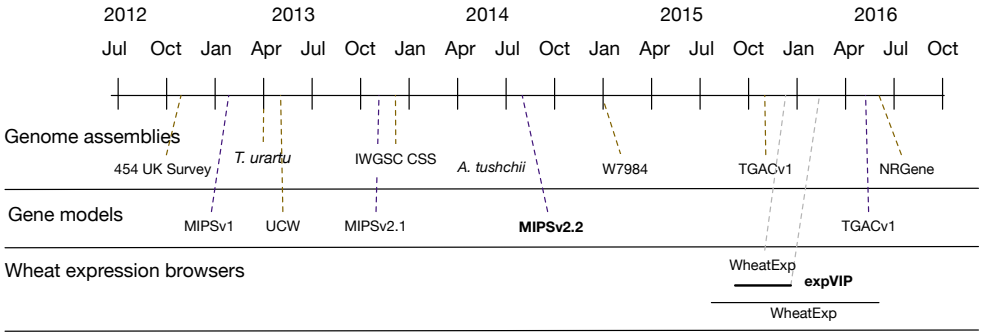


Figure 3.16: Timeline of resources used, or potentially used for *Yr15*.

the decision of reducing the sequenced space with RNA-Seq was appropriate at the time (Figure 3.16). Unfortunately, one of the shortcomings of using RNA-Seq for calling SNPs is that the coverage is not uniform, and the genes that have low expression do not have enough coverage to allow reliable SNP calling (Section 3.3). If a similar study had to be started today, a better alternative would be to use exome capture in general from a segregating population for any trait, or RenSeq if the target gene is a resistance gene.

The quality and completeness of the reference genome or gene models directly affects the mapping of NGS reads. This is particularly true on polyploid organisms: if one of the homoeologues is absent, the reads are likely to map to the wrong genome if the parameters of the aligner are relaxed, or not map at all if the required identity is too high. When the bioinformatic analysis of this project started, the only available wheat genomic reference was a whole genome shotgun 454 sequencing, unassembled (Brenchley et al., 2012); the CSS assembly was being finished (Mayer et al., 2014); the longer scaffolds from Chapman et al. (2015) were not public yet; and finally, the efforts to make a whole genome shotgun assembly were being planned independently by the International Wheat Genome Sequencing consortium (Pozniak, 2016) and TGAC (Clavijo et al. 2016 ; Figure 3.16). Because a contiguous assembly with the corresponding annotation was not available at the time of the analysis, and the fact that all the data available was derived from transcriptomic sequencing, the use of gene models as a reference for the alignment was a suitable approach.

In terms of available gene sets when the analysis started, the canonical reference was the UniGenes from the NCBI (Pontius et al., 2002). The UniGenes are produced with an automated pipeline that clusters all the

Encoding Sequence Tags (ESTs) deposited in the NCBI by identity and selects the longest transcript, which can merge homoeologous transcripts as a single reference. Shortly after I started the bioinformatic analysis, two additional gene models were made available, the draft annotation for the CSS assembly (MIPSv1) in January 2013 and the UCW gene models (Krasileva et al., 2013) in May 2013. I selected the UCW gene models, as they were more mature, phased to distinguish between genomes and already published, over the MIPSv1 genes, as they were still being refined from an initial approach of lifting proteins from related organisms combined with few RNA-Seq experiments. The MIPS gene models were improved by removing duplications in the assembly in a later stage and the nomenclature before the release of the assembly (Mayer et al., 2014), but at that point the results of this project had already been submitted for publication (Figure 1.4; Ramirez-Gonzalez et al. 2015c).

To locate the gene models in the chromosome arms and see if there was an enrichment on the called SNPs, the use of a high resolution consensus map is needed, as the genome assemblies available during the analysis are fragmented. Initially, I used barley to locate the gene models because the genetic map used to locate the CSS scaffolds was not released yet and barley has a conserved synteny across the wheat genomes. The release of a genetic map with over 42,000 markers (Wang et al., 2014) was extremely timely, as it happened during the last phase of the project. Furthermore, as I collaborated in the project, I was also able to use it to locate several CSS scaffolds before the release of the assembly. The located scaffolds were used as proxy to sort just under half of the reference genes in their chromosomal position (Section 3.6). Despite the resolution not being enough to find a single point of enrichment, it was enough to confirm that the SNPs were in the expected location, including one of the SNP candidates flanking the *Yr15* locus (SNP R11, Figure 3.9b). If the analysis was to be done today, the genetic map from Chapman et al. (2015) along with their longer scaffolds, or the scaffolds from TGACv1 or the NRGene should provide a better resolution. Even without having all the CSS scaffolds sorted, the fact that they come from individual chromosome arms enabled the assignment of the genes to a chromosome.

The original expectation was to have a NIL for the BSA to simplify the SNP discovery and analysis since the majority, if indeed not all of the SNPs should be restricted to the region immediately flanking Yr15.

SNP haplotype			Reaction to *P. striiformis*		
R11	R5	R8	Resistant	Intermediate	Susceptible
C	A	T	-	6	16
T	A	T	-	11	-
T	G	C	79	1	-

Figure 3.17: Haplotype analysis and phenotypic evaluation of the 113 doubled haploid lines used in the study. The TGC haplotype corresponds to that originally identified in the *Yr15* parent and which was diagnostic across 112 of the 113 lines studied. Figure from (Ramirez-Gonzalez et al., 2015c)

However the number of SNPs called in the progenitors suggested that the background, Avocet S, was not the same. This happened because despite both susceptible lines being called the same and having the same response to the pathogen, they are in fact different lines from different countries (Section 3.4). This highlights the importance of genotyping the material used when developing mapping populations, especially if the seeds come from different seed banks.

Despite these shortcomings, the use of the BFRs to score the putative SNPs was effective, as most of the SNPs with a high score mapped in chromosome 1B, in line with previous studies ($BFR > 6$, Section 3.7). Using the extra criteria of only selecting SNPs from the resistant progenitor and in the expected chromosome arm, I was able to produce a high resolution genetic map (Section 3.9). The genetic map was of the expected resolution for the size of the population (0.26cM on 196 individuals). Since the mapping population contained only one critical recombinant between *Yr15* and the flanking markers, the population could not yield a better map. To improve the map, a cross from the two critical recombinants could be used to repeat a similar analysis, sequencing with either exome capture or RenSeq.

As described in Ramirez-Gonzalez et al. (2015c):

> The markers R11, R5 and R8 were tested across 122 doubled haploid (DH) lines. These DH lines were derived from crosses crosses between five different UK varieties/breeding lines to *Yr15* derivatives known to carry the resistance gene. The expected *Yr15* haplotype corresponded to T, G and C alleles at markers R11, R5 and R8, respectively (TGC haplotype). The DH lines were tested

at seedling stage for reaction to *P. striiformis*, with 84 showing complete resistance and 34 presenting an intermediate or completely susceptible reaction. The resistant lines all carried the complete *Yr15* haplotype (TGC, Figure 3.17) across the three SNP markers with the exception of five lines which had a single missing data point, but were otherwise consistent. This compared favourably with the most diagnostic in-house SNP markers available within the breeding programmes. Using the three in-house markers, 79 resistant lines carried the expected haplotype, but five completely resistant DH lines were scored as false negative due to the presence of the non-*Yr15* haplotype. Within the intermediate and susceptible DH lines, all but one had a non-*Yr15* haplotype (CAT or TAT) across R11, R5 and R8 (Figure 3.17). This single DH line was scored as a false positive as it carried the TGC *Yr15* haplotype, but was found to have an intermediate (chlorotic) reaction to *P. striiformis*. This line was also the only one scored as a false positive using the three in-house markers.

The fact that the developed markers perform better than markers developed by breeders shows the value of this particular experiment and further confirms that BSA combined with NGS is an effective way to develop novel markers.

In this chapter the integration of different levels of data helped to improve the selection of the candidate SNPs. The main criteria for selecting SNPs was the BFR score. Thanks to the genetic map from Wang et al. (2014) and the CSS scaffolds from Mayer et al. (2014), we were able to confirm that the high scoring SNPs were in the expected region. As the reference genome for wheat improves, defining the location of SNPs linked to a trait of interest will become easier. With a continuous reference between two markers flanking a locus and an improved annotation, it will also be possible to compile a more focused set of candidate genes.

Chapter 4

expVIP: a customisable RNA-seq data analysis and visualisation platform.

4.1 Background.

4.1.1 Alternative expression browsers.

To the best of my knowledge, the only alternative expression browser developed for specifically for wheat is WheatExp (Pearce et al., 2015b). This expression browser contains information of 6 studies from diploid, tetraploid and hexaploid wheat. The studies were selected to be complementary among them; "a broad study of five different tissues across multiple time points (Choulet et al., 2014), a study of seedling photomorphogenesis (Fox et al., 2014), a study of drought and heat stress in wheat seedlings (Liu et al., 2015), a study of wheat grain layers at a single time point (Pearce et al., 2015a), a senescing leaf time-course (Pearce et al., 2014) and a time-course of different grain tissue layers during development (Pfeifer et al., 2014)". The expression quantification is produced by aligning to the gene transcripts using BWA (Li and Durbin, 2010), and the counts are extracted with HTSeq (Anders et al., 2015). The Expression Atlas from EBI is a public resource that collects expression experiments of several species (Petryszak et al., 2016). The samples are processed automatically from the reads deposited ArrayExpress (Kolesnikov et al., 2015), containing data from microarrays and RNA-Seq. The studies included are manually curated and annotated

with the relevant ontologies. As of September 2016, 202 differential and 82 base experiments had been included. The visualisation is designed to explore each gene individually, showing all the related ontologies and details on the expression by factor, or by pairs of factor on a heatmap. The quantification is calculated with HTSeq (Anders et al., 2015), from alignments produced with Tophat (Ling et al., 2013).

4.1.2 Aims

The aims of expression Visualisation and Integration Platform (expVIP) are to:

1. Integrate RNA-Seq experiments from several sources in a single database (Section 4.3).

2. Automate the calculation of the expression values and load them in to the database (Section 4.4).

3. Produce a tool visualisation for said expression values with a short time to learn, good performance, memorability, accuracy and satisfaction s (Section 4.5).

4. Make the system available to the community (Section 4.5).

The software developed in this chapter is published in Borrill, Ramirez-Gonzalez, and Uauy (2016).

4.1.3 Public wheat RNA-Seq experiments.

Table 4.1 contains the 16 studies used during the development of the expVIP. This studies were categorised by developmental time courses, tissues, pathogen infections, and abiotic stresses. The use of divers studies demonstrate the utility of an integrated platform to generate novel hypotheses.

4.1.4 Expression quantification with Kallisto.

Differential expression experiments try to elucidate which genes change under different conditions. To do that, a quantification of the levels of expressions is needed. When using RNA-Seq, the expression analysis usually consists of: aligning the reads to the genome or transcriptome

Table 4.1: Studies with RNA-Seq replicates, sequenced with Illumina, at the time when expVIP was under development.

Study Id	Summary of study	Brief SRA description	Manuscript
DRP000768	Phosphate starvation in roots and shoots	Transcriptome profiles of wheat variety Chinese Spring (CS) in response to Pi starvation (-P) for 10 days.	Characterisation of the wheat (*Triticum aestivum L.*) transcriptome by *de novo* assembly for the discovery of phosphate starvation-responsive genes: gene expression in Pi-stressed wheat (Oono et al., 2013).
ERP003465	fusarium head blight infected spikelets	Near isogenic wheat lines, differing in the presence of the *Fusarium graminearum* FHB-resistance QTL Fhb1 and Qfhs.ifa-5A, under disease pressure (30 and 50 hai) as well as with mock-inoculation	Quantitative trait loci-dependent analysis of a gene co-expression network associated with Fusarium head blight resistance in bread wheat *Triticum aestivum L.*. (Kugler et al., 2013).
ERP004505	grain tissue-specific developmental timecourse	Analysis of the cell type specific expression of homeologous genes in the developing wheat grain	Genome interplay in the grain transcriptome of hexaploid bread wheat (Pfeifer et al., 2014).
SRP004884	flag leaf down regulation of GPC	Wild type bread wheat plants and GPC RNAi plants 12 days after anthesis	Effect of the down-regulation of the high Grain Protein Content (GPC) genes on the wheat transcriptome during monocarpic senescence (Cantu et al., 2011b).
SRP013449	grain tissue-specific developmental timecourse	Transcriptomes of the aleurone and starchy endosperm tissues of the wheat seed (*Triticum aestivum*) at time points critical to the development of the aleurone layer of 6, 9 and 14 days post anthesis.	Gene expression in the developing aleurone and starchy endosperm of wheat (Gillies et al., 2012).
SRP017303	stripe rust infected seedlings	Pool of stripe rust infected wheat leaves	Genome analyses of the wheat yellow (stripe) rust pathogen *Puccinia striiformis f. sp. tritici* reveal polymorphic and haustorial expressed secreted proteins as candidate effectors (Cantu et al., 2013).
SRP022869	*Septoria tritici* infected seedlings	Molecular mechanisms underlying the interplay between fungal pathogenicity and host responses at specific growth phases and the factors triggering disease transition.	Transcriptional Reprogramming of Wheat and the Hemibiotrophic Pathogen *Septoria tritici* during Two Phases of the Compatible Interaction (Yang et al., 2013).

Study Id	Summary of study	Brief SRA description	Manuscript
SRP028357	shoots and leaves of nulli tetra group 1 and group 5	RNA-seq of nulli-tetrasomic wheat lines (shoots and leaves	Patterns of homoeologous gene expression shown by RNA sequencing in hexaploid bread wheat (Leach et al., 2014).
SRP029372	grain tissue-specific developmental timecourse	Gene expression profiling of morphological stage of developing wheat grain	Evaluation of Assembly Strategies Using RNA-Seq Data Associated with Grain Development of Wheat (*Triticum aestivum L.*; Li et al. 2013).
SRP038912	comparison of stamen, pistil and pistilloidy expression	Transcriptional profiling of pistillody stamen, pistil and stamen in wheat line HTS-1	Pistillody mutant reveals key insights into stamen and pistil development in wheat (*Triticum aestivum L.*; Yang et al. 2015).
SRP041017	stripe rust and powdery mildew timecourse of infection in seedlings	Transcriptome Divergence and Overlap for Wheat in Response to Stripe rust and Powdery Mildew Pathogen Stress	Large-scale transcriptome comparison reveals distinct gene activations in wheat responding to stripe rust and powdery mildew. (Zhang et al., 2014).
SRP041022	developmental time-course of synthetic hexaploid	RNA-seq of three tissues of nascent allohexaploid wheat and its following generations, their progenitors, and Chinese Spring	mRNA and Small RNA Transcriptomes Reveal Insights into Dynamic Homoeolog Regulation of Allopolyploid Heterosis in Nascent Hexaploid Wheat (Li et al., 2014)).
ERP008767	grain tissue-specific expression at 12 days post anthesis	Inner pericarp, outer pericarp and endosperm layers from developing grain of bread wheat cv. Holdfast at 12 days post-anthesis.	Heterologous expression and transcript analysis of gibberellin biosynthetic genes of grasses reveals novel functionality in the *GA3ox* family (Pearce et al., 2015a).
SRP045409	drought and heat stress time-course in seedlings	RNA-seq of 1-week old wheat seedling leaves subjected to drought stress, heat stress and their combination before (0h) and after stress (1h or 6h)	Temporal transcriptome profiling reveals expression partitioning of homeologous genes contributing to heat and drought acclimation in wheat (*Triticum aestivum L.*; Liu et al. 2015).
INRA-RNAseq (ERP004714)	developmental time-course of Chinese Spring	Whole transcriptome sequencing of wheat 3B chromosome	Structural and functional partitioning of bread wheat chromosome 3B (Choulet et al., 2014).
SRP056412	grain developmental timecourse with 4A dormancy QTL	This study was to identify candidate genes underlying the 4AL QTL for grain dormancy in wheat. RNA was sequenced from pooled NILs segregating for the QTL	Transcriptomic analysis of wheat near-isogenic lines identifies *PM19-A1* an *A2* as candidates for a major dormancy QTL (Barrero et al., 2015)

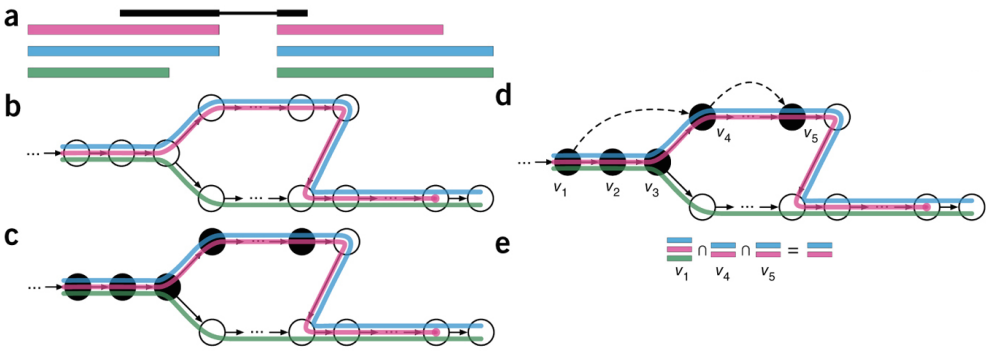


Figure 4.1: "Overview of Kallisto. The input consists of a reference transcriptome and reads from an RNA-seq experiment. (a) An example of a read (in black) and three overlapping transcripts with exonic regions as shown. (b) An index is constructed by creating the transcriptome de Bruijn Graph (T-DBG) where nodes (v1, v2, v3, ...) are k-mers, each transcript corresponds to a coloured path as shown and the path cover of the transcriptome induces a k-compatibility class for each k-mer. (c) Conceptually, the k-mers of a read are hashed (black nodes) to find the k-compatibility class of a read. (d) Skipping (black dashed lines) uses the information stored in the T-DBG to skip k-mers that are redundant because they have the same k-compatibility class. (e) The k-compatibility class of the read is determined by taking the intersection of the k-compatibility classes of its constituent k-mers" (Bray et al., 2016).

reference and quantifying the expression according to how many reads map to a region. However, this process usually takes around 6 hours per sample. Aligners such as `bwa` or `bowtie` produce a detailed alignment of each read, which is useful find polymorphisms (see Chapter 3) or to find novel alternative splices (Trapnell et al., 2012).

For expression analysis only the count of how many reads with a transcript is required, calculating the best local alignment and the output of each read is unnecessary. `Kallisto` is a tool that generates an index based on overlapping k-mers (sequences of size k), which are connected sequentially to represent each transcript on a transcriptome de Bruijn Graph (T-DBG). For alternative splicings of the same gene, were some sequence overlap between transcripts, the connections produce two different sets of connections between k-mers. The k-mers on each read are then used to find the compatible transcripts across the T-DBG and those are counted. Finally, the program estimates the abundance of the transcript in the sample (Figure 4.1; Bray et al. 2016).

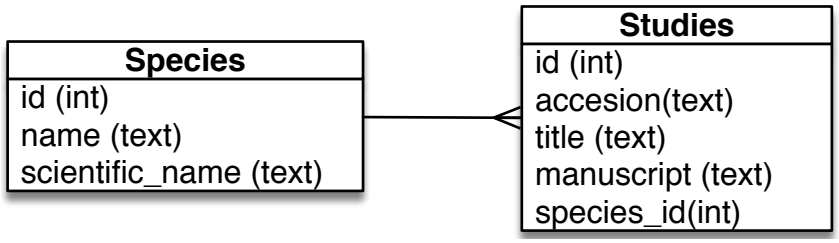


Figure 4.2: Example of a relationship between tables. The tables Species and Studies are related. Each study has one species and each species can have several studies.

Table 4.2: Example content for the table `species`

id	name	scientific_name
1	Bread wheat	Triticum aestivum
2	Yellow rust	Puccinia striiformis
3	wheat and rust	T.aestivum,S.tritici

4.1.5 Relational databases.

A relational database is a set of structured tables that have relationships between each other. The tables correspond to the data that is essential for the represented concept (domain). For example, in a table representing several species, the common name and the scientific name belong to the same domain (ie name: Bread wheat; scientific name *Triticum aestivum*). Tables in the same relational database form relationships between each other. Continuing with the example, a species can have several scientific studies related to them. The domain of a study can be formed by the accession, a title, a corresponding manuscript and the species it is concerned with. The two tables can be connected by the usage of a common ID signifying the species. It is therefore common practice to create an extra column containing unique integer values, to act as connecting keys between two or more tables. Such a column is defined as the primary key of the table; although, strictly speaking, any set of columns whose combination of values is unique for each row can act as primary key. In our example an extra `id` column is added allowing to create a stable relationship between the `Species` and the `Studies` tables. (Figure 4.2; Codd 1970). The tables 4.2 and 4.3 have the content of their corresponding domains.

Table 4.3: Example content for the table `studies`

id	accession	manuscript	species_id
1	DRP000768	10.1186/1471-2164-14-77	1
2	ERP003465	10.1186/1471-2164-14-728	1
3	ERP004505	10.1126/science.1250091	1
4	SRP004884	10.1186/1471-2164-12-492	1
5	SRP013449	10.1111/j.1467-7652.2012.00705.x	1
6	SRP017303	10.1186/1471-2164-14-270	2
7	SRP022869	10.1371/journal.pone.0081606	3

4.1.6 SQL.

Standard Query Language (SQL) is a common language to retrieve information from relational databases. SQL provides operations to select columns and rows, join tables, group repeated values and order the results. Those simple operations are enough to retrieve information between tables (Oracle, 2014). The following list shows a brief description of some commands that can be used to build a query.

`SELECT <EXPRESSIONS>` A list of columns or an expression that will be displayed, separated by commas (`,`). To display all the columns, the `*` character all the columns in the table. The order of the columns will be the same as the order given in this part of the command

`FROM <TABLE>` follows the column names to add a list of tables to select from.

`JOIN <TABLE> ON <EXPRESSION>` is used to join the table from the left side of the statement with the `<EXPRESSION>` given after the `ON` clause.

`WHERE <EXPRESSION>` filters the rows by the `<EXPRESSION>`

`ORDER BY <COLUMNS>` The rows will be sorted by the natural order of the given `<COLUMNS>`.

`GROUP BY <COLUMNS>` The rows are merged by the columns stated. This can be used to get an unique set of values and apply a function to all the rows that have the same value, as a count.

Expressions can be values, operators or functions like:

`COLUMN` The value of a column.

`<EXPRESSION> = <EXPRESSION>` `TRUE` when both, the left and right, `<EXPRESSION>` are equal. `FALSE` otherwise

`<EXPRESSION> > <EXPRESSION>` `TRUE` when the left `<EXPRESSION>` is greater than the right `<EXPRESSION>` are equal. `FALSE` otherwise

`<EXPRESSION> < <EXPRESSION>` `TRUE` when the left `<EXPRESSION>` is less than the right `<EXPRESSION>` are equal. `FALSE` otherwise

`COUNT(*)` The count of rows that have the same values, as selected in the `GROUP BY` clause.

A simple query to join the `species` and `studies` tables and displaying only the species name, scientific name and accession of the study is shown in Listing 4.1. The results of the query are in Table 4.4.

Listing 4.1: Join example query

```
SELECT
  species.name,
  species.scientific_name,
  studies.accession,
FROM species
JOIN studies on species.id = studies.species_id;
```

Table 4.4: Join of the `species` and `studies` table.

name	scientific_name	accession
Bread wheat	Triticum aestivum	DRP000768
Bread wheat	Triticum aestivum	ERP003465
Bread wheat	Triticum aestivum	ERP004505
Bread wheat	Triticum aestivum	SRP004884
Bread wheat	Triticum aestivum	SRP013449
Yellow rust	Puccinia striiformis	SRP017303
wheat and rust	T.aestivum,S.tritici	SRP022869

The relationships between tables can be of the following types:

one-to-one. When rows on a table can be related to a row in a second table. On the diagrams they are represented by a straight line.

many-to-many. Rows on a table can have many corresponding rows in a second table, resented with lines with whiskers on both sides of the line.

one-to-many. Rows on a table can be related to many rows on the second table, represented with whiskers only on one side of the line.

An important feature of a database is the ability to store the data consistently. A transaction is a set of related operations that need to be performed at the same time; as such, it needs to follow the principles of Atomicity, Consistency, Isolation and Durability (ACID) (Haerder and Reuter, 1983).

Atomicity. All the operations or none have to be performed. If any of them fails or an error happens while the transaction is executed, the data has to be restored to the original status.

Consistency. The changes in the database have to be valid before and after the transaction.

Isolation. If more than one transaction is being executed at the same time, the result must be the same as if the transactions were executed one after the other.

Durability. The result of the transaction is stored even if the server is restarted.

Several Relational Database Management System (RDBMS) implement SQL, with various levels of compliance to the standard and different licenses. A popular RDBMS is `MySQL`. From the beginning `MySQL` aimed to be a lightweight and easy to install open source product (Oracle, 2014). This characteristics made it popular on the web and it is currently the RDBMS behind Ensembl! (Flicek et al., 2012).

4.1.7 Model-View-Controller

The Model View Controller (MVC) is a metaphor to isolate the user interactions from the underlying data. The models hold the data on logical their domains. The views contain the layout on how the models are displayed to the user. The controllers receive the requests from the users

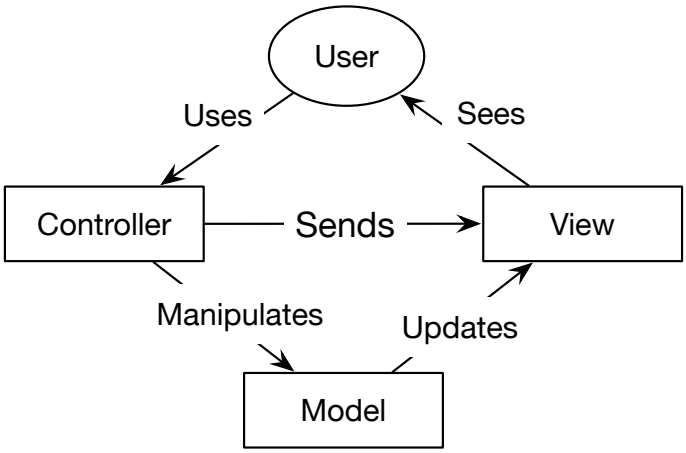


Figure 4.3: MVC interaction between components.

and modify the models hold the data as mapped to their logical domains accordingly and send a view back for display. The MVC metaphor allows the development of independent parts of the system and helps to structure the underlying representation of the domains. (Figure 4.3; Krasner and Pope 1988).

Ruby on Rails (RoR) is a framework to develop web applications heavily influenced by the MVC metaphor. It is based on the Rails language and provides several tools designed to facilitate development, such as automated tasks designed to create models with their corresponding views and controllers. On the top of that, it provides the tools to manage the connection and queries to the RDBMS, allowing the developer to focus on functionality (Rails Guide, 2016).

4.1.8 Data visualisation

In the last couple of decades the amount of information available in any given field has been growing exponentially, in part thanks to the internet. A standing challenge is therefore to produce tools that help interpretation. An effective way to communicate large amount of data is through visualisation, but it has to have the following properties to be usable (Myatt and Johnson, 2011):

Time to learn. The user needs to take little time to learn how to use the system to extract the information they need. Also, all the features should be easy to find.

Performance. The tool needs to be quick to access and transform the visualisation as the user requests.

Accuracy. The tool has to perform as the user expects, so if the tool is prone to make users commit mistakes, it is not accurate.

Memorability. Once the user learns to use the system, is it easy to remember how to use it? Systems that change their interface often, or between windows are not as easy to remember.

Satisfaction. The user responds positively to the use of the system and the time is spent actually exploring the data, rather than trying to make the system work.

4.2 General design

One of the main objectives of expVIP is to make the public expression datasets easily accessible and explorable for the target community (currently wheat, but not limited to it). A web interface is an effective way to reach a global audience. A web service requires to have a server to run the application, and a browser to connect to the server and display the application to the user (ie Internet Explorer, Chrome, Firefox). The web server technology used for expVIP is RoR, as it abstracts the MVC metaphor and it is designed to speed development (Rails Guide, 2016). In order to display the expression data to the users, expVIP relies on a BioJS component (Yachdav et al. 2015, Section 4.5) developed for expVIP. All the data is stored in a MySQL database (Section 4.3) and it is accessed through models developed under RoR (Figure 4.4).

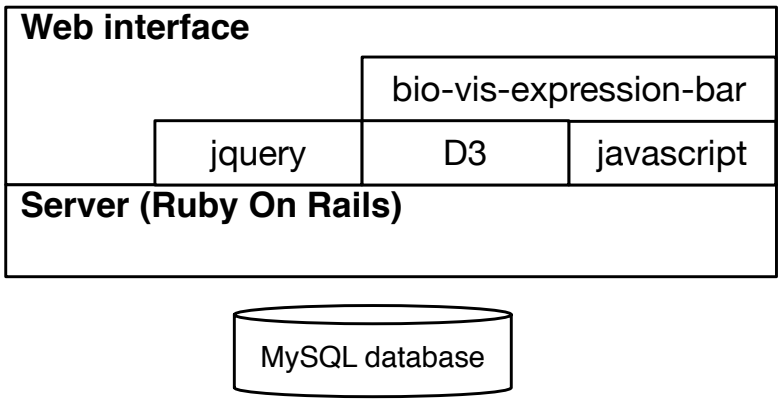


Figure 4.4: General design of expVIP

4.3 Database design

To address the different types of conditions over different experiments, expVIP is designed around a relational database. The design comprises two core groups of tables and two auxiliary tables that take care of different species and homoeologues (Figure 4.5).

Metadata The tables in this group contain the information of each one of the studies.

- **Studies** holds general information for a study, which contain several experiments. The table also contains the reference to the paper where the data is published and the accession number for the study.
- **Experiment group** keeps together all the individual experiments that come from the same study and that were taken on the same condition (ie. replicates).
- **Factors** holds all the possible factors used to group the experiments. Each experiment group has many factors and each factor group has many experiment groups. As the experiment does not have a fixed number of columns representing each factor, it is possible to have any arbitrary number factors to group.
- **Experiment** holds the information of each individual experiment, with the corresponding accession.

Expression values. The tables on this block contain the information for each genes and their expression values.

- **Types of value** keeps a list of different units that are stored. On the original design TPM and raw counts are set up, but as the units are not hard coded it is possible to use FPKM, RPKM or any other unit.
- **Gene Set** contains the name of a reference gene set for the analysis. On the original version of expVIP, the gene models from the IWGSC as deposited in Ensembl release 26 were used (Mayer et al., 2014). However, this table allows to use several reference gene models on the same database.

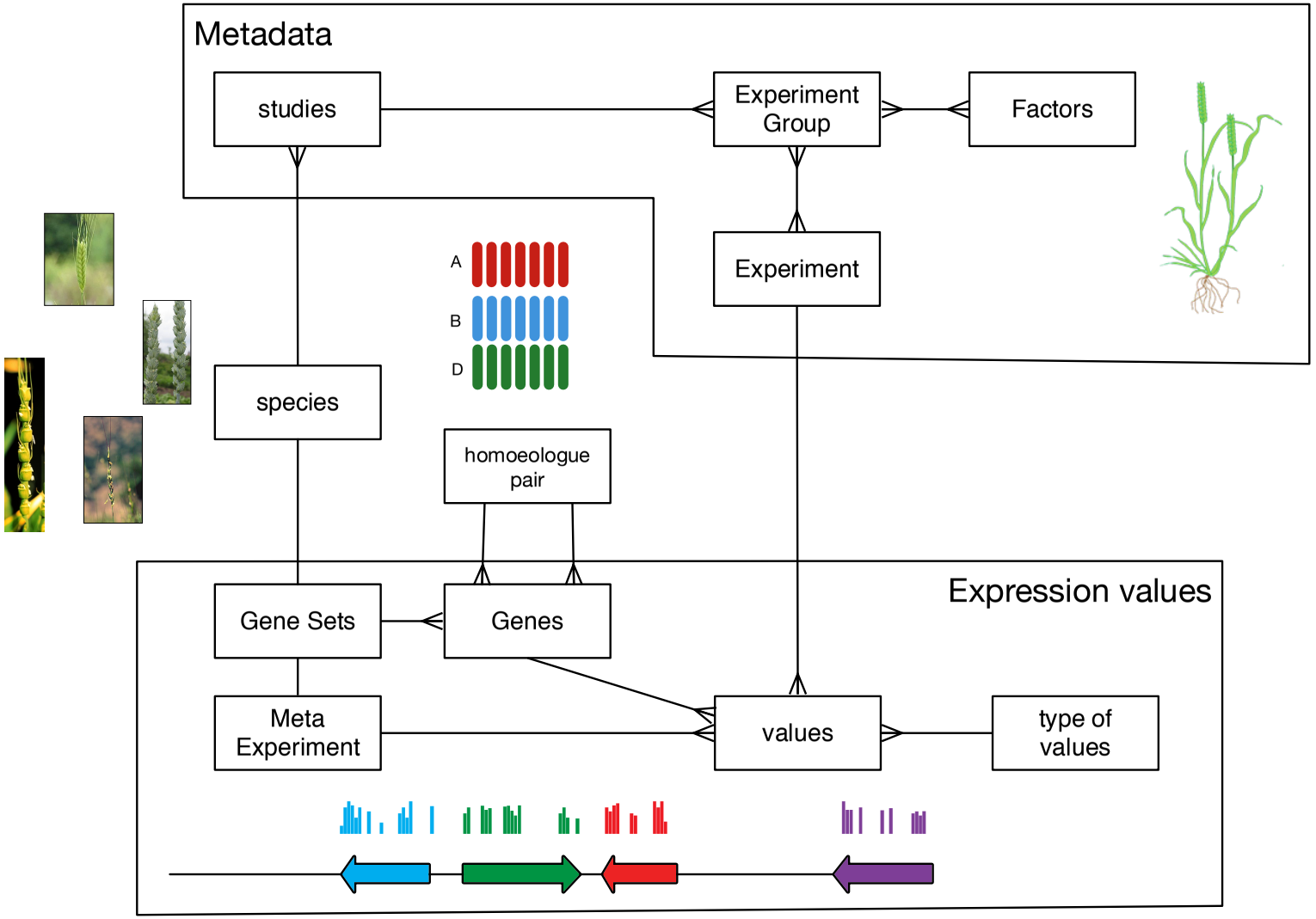


Figure 4.5: Database design. The block on the top stores the meta-data about the experiments and the studies. The bottom block consist on the tables related to the expression values. Species and homoeologues are outside the main blocks as they are not core to the groups. The whiskers in the connections show the cardinality of the relationships.

Genes are related to a `gene set`, so even if two genes coming from two different datasets share the same name it is possible to distinguish them and avoid ID collisions. This situation is unlikely to occur when using published references, but might arise when joining several *de novo* gene model datasets.

Meta Experiment allows to have the same data analysed with different tools. By default expVIP uses Kallisto (Bray et al., 2016). However other tools, or different versions of the same tool, can be used to repeat the analysis.

Values have a domain that includes the `meta experiment`, `gene` and, `type of value`.

Homoeologues contains the relationship between genes. This allows to get the expression values of several related genes.

Species contains the target species of a study. It is not linked to the gene models to allow the direct comparison between related species using the same gene models (ie, *T. aestivum* vs *T. turgidum*).

In the cases where a relationship between tables is not unique, such as `experiment_groups` having many `factors` and the `factors` having many `experiment_groups`, storing of relationships is done with an auxiliary table (for example, `ExperimentGroups_Factors`, not explicitly shown in Figure 4.5, but implicit by the lines with whiskers).

Once all the data is stored, the tables can be queried together to make clear the relationship between specific rows. One of the core tasks of expVIP is to get all the factors that define each experiment, in order to be able to merge similar studies. To retrieve the `experiments` and `factors` of an `experiment group`, auxiliary tables `ExperimentGroups_Factors` and `experiment_groups_experiments` are used in the query. (Listing 4.2 and Table 4.5).

Listing 4.2: Query experiments and factorsQuery experiments and factors from accession 'DRR003148'

```
SELECT
  experiments.accession,
  factors.factor,
  factors.description,
  experiment_groups.name as experiment_group
FROM factors
JOIN ExperimentGroups_Factors
  ON factors.id = ExperimentGroups_Factors.factor_id
JOIN experiment_groups
  ON experiment_groups.id = ExperimentGroups_Factors.
     experiment_group_id
JOIN experiment_groups_experiments
  ON experiment_groups_experiments.experiment_group_id =
      experiment_groups.id
JOIN experiments
  ON experiments.id = experiment_groups_experiments.
     experiment_id
WHERE accession =  'DRR003148'
```

Table 4.5: Results of querying the metadata for accession 'DRR003148' (Listing 4.2)

accession	factor	description	experiment group
DRR003148	Age	24 days	Group1
DRR003148	High level age	vegetative	Group1
DRR003148	High level stress-disease	no stress	Group1
DRR003148	High level tissue	roots	Group1
DRR003148	High level variety	Chinese Spring	Group1
DRR003148	Stress-disease	none	Group1
DRR003148	Tissue	roots	Group1
DRR003148	Variety	Chinese Spring	Group1

Likewise, to get the `expression_values` for a `gene` with the corresponding unit (`type_of_values`) and `experiment` a simple query joining the four tables is used. The Listing 4.3 retrieves the `expression_values` for the `gene` 'Traes_5BS_0AFC3F795.1', and the result is on Listing 4.6

Listing 4.3: Query values from 'Group1' and gene 'Traes_5BS_0AFC3F795.1'

```
SELECT
  genes.name as gene,
  expression_values.value,
  experiments.accession,
  type_of_values.name as unit
FROM expression_values
JOIN genes
  ON expression_values.gene_id = genes.id
JOIN type_of_values
  ON type_of_values.id = expression_values.
     type_of_value_id
JOIN experiments
  ON experiments.id = expression_values.experiment_id
WHERE
  genes.name = 'Traes_5BS_0AFC3F795.1'
```

Table 4.6: Results of query to get the values for gene 'Traes_5BS_0AFC3F795.1' (Listing 4.3), only 'Group1' is displayed from the output. The three values with the same unit correspond to replicates on the same study.

gene	value	accession	experiment group	unit
Traes_5BS_0AFC3F795.1	136.995	DRR003148	Group1	count
Traes_5BS_0AFC3F795.1	120.683	DRR003149	Group1	count
Traes_5BS_0AFC3F795.1	140.94	DRR003150	Group1	count
Traes_5BS_0AFC3F795.1	24.2277	DRR003148	Group1	tpm
Traes_5BS_0AFC3F795.1	23.9739	DRR003149	Group1	tpm
Traes_5BS_0AFC3F795.1	24.9835	DRR003150	Group1	tpm

With those two queries is enough to retrieve all the information required to do sub-groupings.

The database is implemented using the RDBMS `MySQL 5.5`.

4.4 Data integration pipeline

To prepare the database, expVIP requires to have all the metadata for the experiments to integrate. ExpVIP contains tasks to load all the metadata and a wrapper for Kallisto that can be run from expVIP. Alternatively,

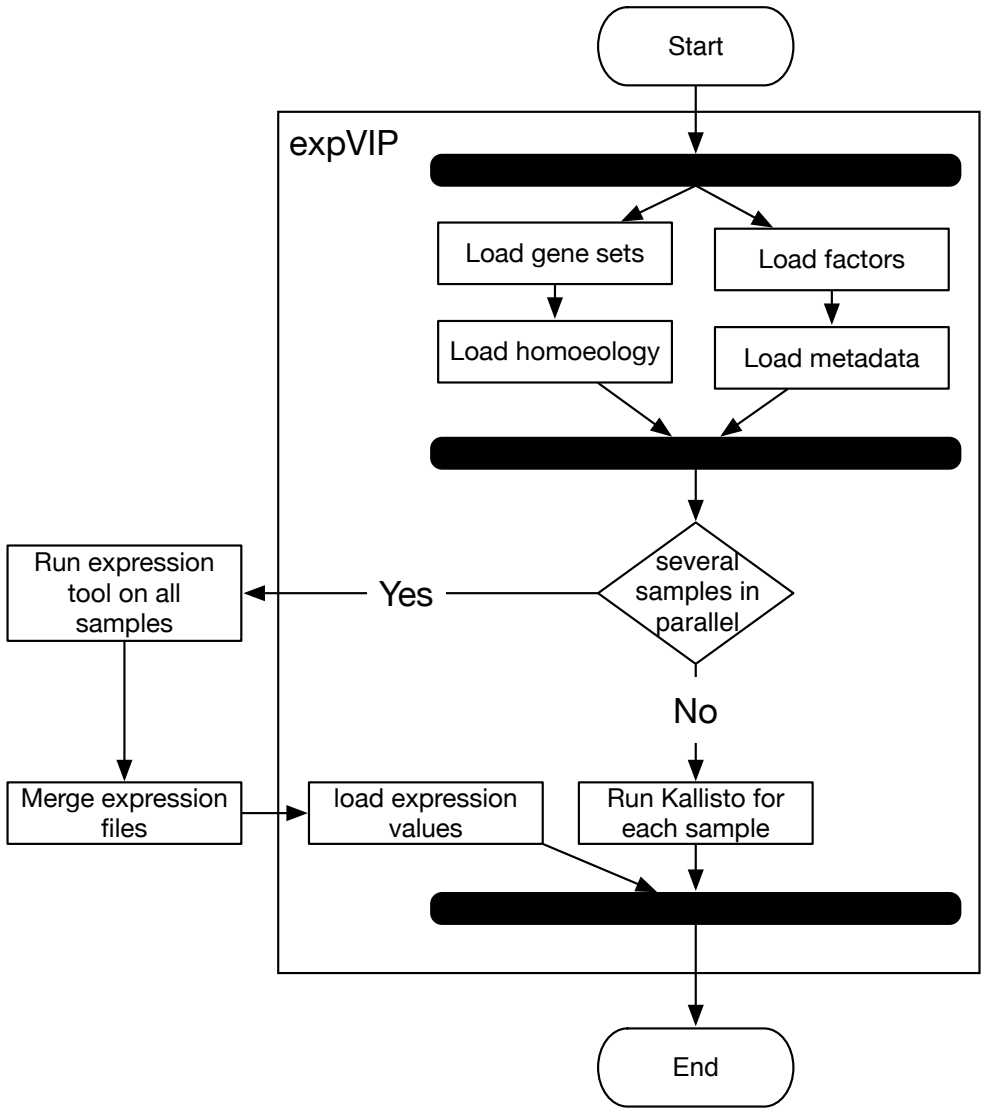


Figure 4.6: The pipeline for loading the data into expVIP. The black lines represent a border of tasks that are not required to be executed in a particular order.

the expression values can be calculated with another tool and loaded as a single file, this approach is preferred for a large set of samples (Figure 4.6). Details on how to load the files in the database are in the expVIP tutorial (Appendix C).

The required files for the metadata are:

Factors. The file contains all the possible factors that can be used to group all the experiments. The file must contain the following columns (Table 4.7):

Table 4.7: Factors file. The table must be saved as a text file, with columns separated by tabs

factor	order	name	short
Age	1	7 days	7d
Age	2	seedling stage	see
Age	3	14 days	14d
Age	4	three leaf stage	3_lea
Age	5	24 days	24d
High level age	1	seedling	see
High level age	2	vegetative	veg
High level age	3	reproductive	repr
High level stress-disease	1	none	none
High level stress-disease	2	disease	dis
High level stress-disease	3	abiotic	abio
High level stress-disease	4	transgenic	trans
High level tissue	1	spike	spike
High level tissue	2	grain	grain
...			

factor The category were the factor belongs. In the case of the initial dataset used in expVIP, the grouping factors are: Age, stress-disease, tissue and a corresponding 'High level' for each factor. The metadata file must contain a column corresponding to each one of this factors.

order The default order in which to display each factor. This ensures that the age of the plants is sorted chronologically.

name Long description of each level for the category. The values in this column must match the values in the metadata file (see below).

short Is a short name, used when the space to display the full description of the factor is not enough.

metadata The metadata file is the file that contains the information related to each study and the corresponding experiments. Each study contains several experiment groups (replicates), which in turn contain every individual experiment. The factors must be shared across experimental groups.

secondary_study_accession The accession number for experiments carried as part of a single study. This is usually the high level BioProject or SRA number.

run_accession The accession of the individual run.

scientific_name of the species.

experiment_title A description for the individual RNA-seq sample.

study_title A description of the general study.

Manuscript The DOI of the study.

Group_for_averaging A description of the experiment. This must be the same all the replicates in the same study.

Group_number_for_averaging A short name for replicated experiments.

Total reads (optional)

Mapped reads (optional)

Besides the main fields, each factor has a a corresponding column Variety, Tissue,Age, Stress-disease, High level variety, High level tissue, High level age and High level stress-disease

Gene set The gene set is provided as a single `fasta` file. The file may contain alternative transcripts from the same gene. To identify this, the `fasta` header may include the optional fields `gene` and `transcript`. In the absence of this, the only stored value is the name derived from the header, going from the '>' character to the first space on the line (Listing 4.4).

Listing 4.4: A fasta entry on of the gene set.

```
1 >Traes_5BL_3FC5BA305.1 cdna:novel scaffold:IWGSC2:
    IWGSC_CSS_5BL_scaff_1082268:5:199:-1 gene:
    Traes_5BL_3FC5BA305 transcript:
    Traes_5BL_3FC5BA305.1
2 TGCTGCTGCTAGGCTTGAAGAGGTTGCTGGCAAGCTCCAGTCTGCTC
3 GGCAGCTCATTCAGAGGGGCTGTGAGGAGTGCCCCAAGAACGAGGAT
4 GTTTGGTTCGAGGCATGCCGGTTGGCTAGCCCAGATGAGTCAAAGGC
5 AGTAATTGCCAGGGGTGTGAAGGCAATTCCCAACTCTGTGAAGCTGT
6 GGCTGCA
```

homoeologues A file containing the homoeologues for the A, B and D genomes. Currently these are the only supported default names. The file also includes a column with the gene name and to which Group (ie 1, 2, 3 ... 7) and Genome (ie A, B or D) it belongs (Table 4.8).

expVIP includes several tasks to load the different files. For example, to load the factors the `load_data:factor` starts a transaction (Listing 4.5; line 2) to ensure that all the data is loaded, and if for some reason the load fails, the database is restored to the previous status. In the transaction, the file is read row by row using the `csv` library (line 3). The function `find_or_create_by` is a function that RoR provides on models to create an entry in the table, or update it if already exists. Each row is used to create or update a `Factor` (lines 374-376). A similar strategy is used for all the files that are regular tables.

Listing 4.5: Task that loads factors

```
1 task :factor, [:filename] => :environment do |t, args|
2   ActiveRecord::Base.transaction do
3     CSV.foreach(args[:filename], :headers => true, :
          col_sep => "\t") do |row|
4       factor = Factor.find_or_create_by(:factor=>row["
            factor"], :description=>row["name"], :name=>
            row["short"])
5       factor.order = row["order"].to_i
6       factor.save!
7     end
8   end
9 end
```

The gene sets are loaded slightly differently, as the input is a `fasta` file, as opposed to tabular file. The reader for the `FastaFormat` from BioRuby (Goto et al., 2010) is used to read the file (Listing 4.6; line 4). Since expVIP only records the name of the genes, only the id of the fasta sequence is extracted (lines 6-79). The name is stored in the name and cDNA columns. The parser for entries from Ensembl, such the one in Listing 4.4 include code to load the cDNA and transcript fields correctly.

Table 4.8: Example tabular file containing the homoeology across the three genomes.

Gene	A	B	D	Group	Genome
Traes_5BS_0AFC3F795	Traes_5AS_0AFCC204EBA	Traes_5BS_0AFC3F795	Traes_5DS_C204EBAA9	5	B
Traes_5DS_C204EBAA9	Traes_5DS_0AFCC204EBA	Traes_5BS_0AFC3F795	Traes_5DS_C204EBAA9	5	D
Traes_7DL_82360D4EE1		Traes_7BL_3F7958204	Traes_7DL_82360D4EE1	7	D
Traes_2AL_1368BE0AD	Traes_2AL_1368BE0AD		Traes_2BL_CD459994C1	2	A
...					

Listing 4.6: Task that load genes from a fasta file

```
1 task :de_novo_genes, [:gene_set,:filename] => :
    environment do |t, args|
2   ActiveRecord::Base.transaction do
3     gene_set = GeneSet.find_or_create_by(:name=>args[:
        gene_set])
4     Bio::FlatFile.open(Bio::FastaFormat, args[:filename
        ]) do |ff|
5       ff.each do |entry|
6         arr = entry.definition.split(/\s+/)
7         name = arr[0]
8         g = Gene.new
9         g.gene_set = gene_set
10        g.name = name
11        g.cdna = name
12        g.save!
13      end
14    end
15  end
16 end
```

There are two options to load the expression values from the database: a matrix with all the expression values and running `Kallisto` from expVIP.

The task in Listing 4.7 loads the expression values from a tabular file with the genes as rows and the values as columns. The exception handling and messages are removed The task requires the following arguments:

`meta_experiment`. A name for the analysis. This can be the name of the tool used for the expression quantification combined with the name of the reference, as a single text variable.

`gene_set`. The reference used for the analysis.

`value_type`. The unit of the file (ie. TPM, count)

`filename`. The file that is going to be loaded in the database.

The steps to load the values are:

1. A transaction is initiated at the beginning of the task, to ensure that if any step fails and the execution is aborted the database will stay in a consistent state (Listing 4.7; line 2).

2. The connection is assigned to the variable `conn`, to be able to execute queries directly to the database (line 3).

3. The `meta_experiment`, `gene_set` and `value_type` are loaded and stored to get the corresponding IDs in the insertion (line 4).

4. All the `Genes` and `Experiments` are loaded in their corresponding hash table, to be able to get the IDs when the actual values are inserted (lines 7-11).

5. The file is read with the `CSV` library from Ruby, keeping the headers to be able to assign the correct experiment (line 14).

6. The first column is named `target_id` and contains the gene name. The ID of the gene in the database is retrieved from the previously loaded hash (lines 15-16)

7. Each column is iterated and the values needed to execute the insertion to the database are concatenated.

8. Whenever the number of queued insertions reaches 1,000, the command to perform the insertions is executed (line 25).

9. As the number of genes is not usually a multiple of 1,000, when the process finished reading the file an extra insertion is executed to empty the queue (line 30).

The decision to execute the insertions in batches of 1,000 objects is to reduce the number of processes running in the database, while keeping low the memory usage of the application. This approach is faster than using the functions for insertions RoR on multiple values. For trivial operations, the functions from the framework are used, as they are easier to maintain (compare insertion in line 4 to the block of code from line 18 to 26).

Listing 4.7: Task to load the expression values from a tabular file.

```
task :values, [:meta_experiment, :gene_set, :value_type,
    :filename ] => :environment do |t, args|
ActiveRecord::Base::transaction do
conn = ActiveRecord::Base.connection
meta_exp = MetaExperiment.find_or_create_by(:name=>args
    [:meta_experiment])
gene_set = GeneSet.find_by(:name=>args[:gene_set])
value_type = TypeOfValue.find_or_create_by(:name=>args[:
    value_type])
experiments = Hash.new
meta_exp.gene_set = gene_set
genes = Hash.new
Gene.find_by_sql("SELECT * FROM genes where gene_set_id
    ='#{gene_set.id}'").each {|g| { genes[g.name] = g.id
    }
Experiment.find_each{|e| experiments[e.accession] = e.id
    }
count = 0
inserts = Array.new
CSV.foreach(args[:filename], :headers => true, :col_sep
    => "\t") do |row|
  gene_name = row["target_id"]
  gene = genes[gene_name]
  row.delete("target_id")
  row.to_hash.each_pair do |name, val|
    val = val.to_f
    str = "(#{experiments[name]},#{gene},#{meta_exp.id
       },#{value_type.id},#{val},NOW(),NOW())"
    inserts.push str
  end
  count += 1
  if count % 1000 == 0
    sql = "INSERT INTO expression_values (`experiment_id
       `,`gene_id`, `meta_experiment_id`, `
       type_of_value_id`, `value`,`created_at`, `
       updated_at`) VALUES #{inserts.join(", ")}"
    conn.execute sql
    inserts = Array.new
  end
end
sql = "INSERT INTO expression_values (`experiment_id`,`
    gene_id`, `meta_experiment_id`, `type_of_value_id`,
    `value`,`created_at`, `updated_at`) VALUES #{inserts
    .join(", ")}"
conn.execute sql
end
end
```

Alternatively, expVIP can execute `Kallisto` on all the samples loaded in the database. For this purpose, expVIP stores the raw reads in FastQ format, organised in directories named with the same accessions as in the metadata (ie a directory named `DRR003148` contains the reads for the metadata displayed in table 4.5). expVIP takes all the accessions for the experiments in the database and searches for the corresponding folder. If the folder exists and if it contains the `fastq` files, then it is deemed as valid. If the folder already has the `Kallisto` output, the next folder is evaluated, otherwise `Kallisto` is executed with its default settings and the results are loaded into the database. This process is repeated for all the accessions (Figure 4.7). This pipeline allows to populate the database partially, in case that not all the experiments are ready from the beginning.

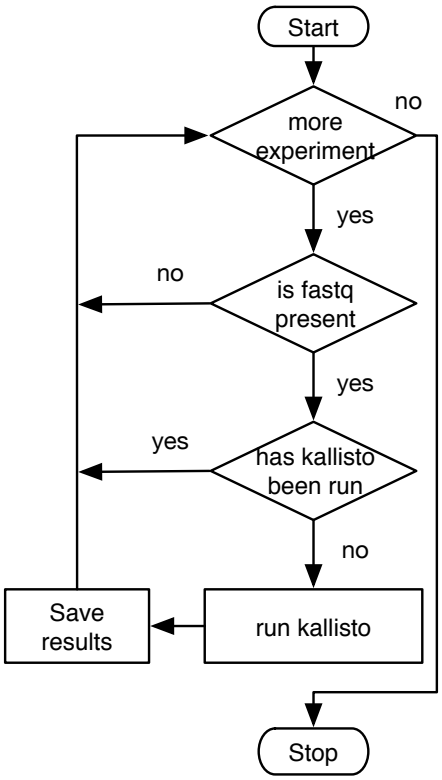


Figure 4.7: Steps to run and load Kallisto

New experiments can be added to the existing metadata file or to a new file; the expVIP loading procedure then can be run again to update the list of experiments and the corresponding expression values. This design allows to keep the database updated as more experiments become available. The fact that the loading is done in transactions ensures that the database is kept consistent, regardless of potential errors in the input files.

4.5 Graphical interface

With the expression across experiments integrated in a single database, the next challenge was to make the data accessible to a wide audience. RoR has tasks to automate the construction of controllers and views from the MVC metaphor, helping on the retrieval and formatting of the raw

data. However, the main objective was to visualise the data from all the experiments as intuitively as possible. To make the visualisation dynamic in a browser, the use of `JavaScript` is necessary, as it is the only widely adopted programming language used for web content. Among the tools built on the top of `JavaScript`, `D3` is a framework designed explicitly to do dynamic visualisations (Bostock et al., 2011).

Usability was a top priority on the design of the visualisation component for expVIP. The time needed to learn, performance are accuracy were taken into account when designing expVIP. As memorability and satisfaction are subjective, they were not directly tested for during development.

Time to learn. The controls are located in two blocks, one for global controls (ie. unit, save plot) and one to modify the factors, close to the factor they affect (Figure 4.8).

Performance. All the data for the genes being displayed is loaded from the database in a single transaction. The data is available in the cache of web browser and whenever the user changes anything in the visualisation, new values are calculated locally.

Accuracy. As knowing what the users will do is not obvious, the visualisation was given to a panel of potential users (other members of the Uauy lab) for comments. In previous versions the legends were a confusing and the location of the buttons was too distant from the aspect of visualisation that they controlled. After reviewing the feedback, the accuracy was improved. Also, the position of the controls don't change, regardless of the representation of the data (bar plot or heatmap).

The elements in the graphical are shown in Figures 4.9, 4.11 and 4.12, with a description of each element of the GUI listed below.

1. **Search box**: at any point it is possible to type a new gene name (based on Ensembl Plants nomenclature) and generate a new set of expression data.

2. **Compare box**: it is possible to add a second gene and press the Compare button to generate two expression graphs drawn at the same scale.

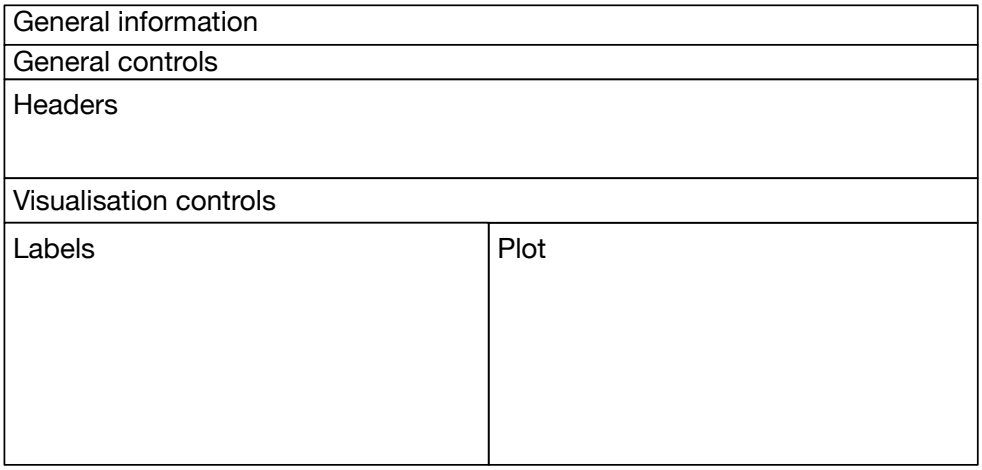


Figure 4.8: expVIP Graphical User Interface components. The top bar shows a short description of what is displayed. The General and Visualisation controls contain the buttons and menus that enable the interaction with the figure. The Headers, Labels and Plot are the actual components of the visualisation

3. **Menu options**: has several links on the details of the study and tutorial. The menu can be edited to customise instances of expVIP.

4. **Gene**: shows the currently displayed gene, with a link to Ensembl to the corresponding description.

5. **Expression unit**: selects the expression unit to visualise. This can be either Transcripts per Million of mapped reads (TPM) or estimated counts. If other units are loaded in the database, they will appear in this field.

6. **Save graph**: these two buttons allow users to save the current graphs in either SVG (to work on Adobe Illustrator) or as PNG files. The export process selects the graphical elements from the visualisation and binds them together on a single `SVG` file. The reasons to follow this process are: to ensure that the plot reflects the user selection; remove the elements that do not have a meaning in a static context and; allow the user to format the image with publication quality (Figure 4.10).

7. **Save data**: downloads a csv file with the data with the current selection and order of factors as displayed on the screen. The data will include the standard errors and the number of samples that make up each value. An example of how the output looks is in Listing 4.8.

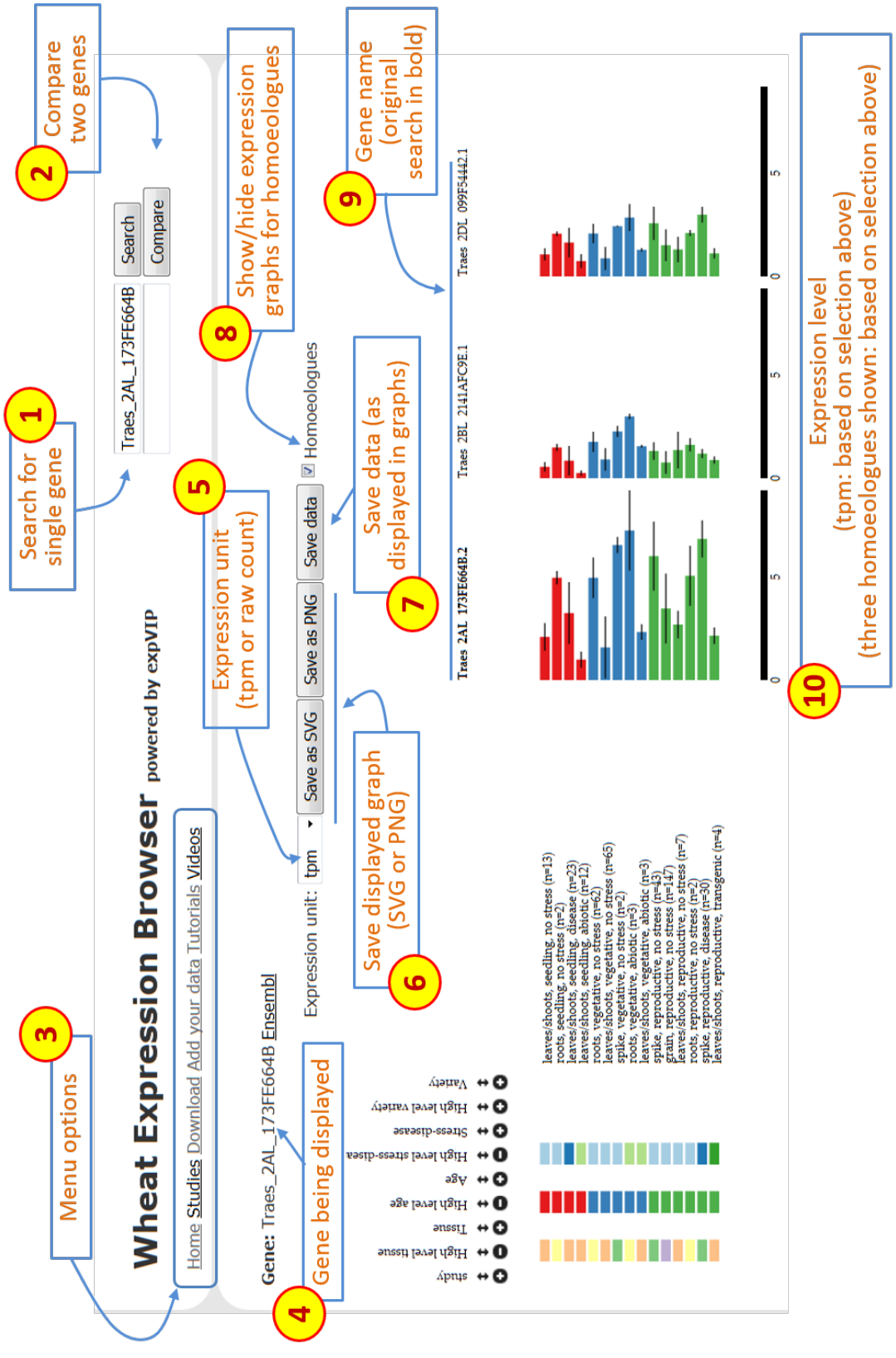


Figure 4.9: Features on expVIP

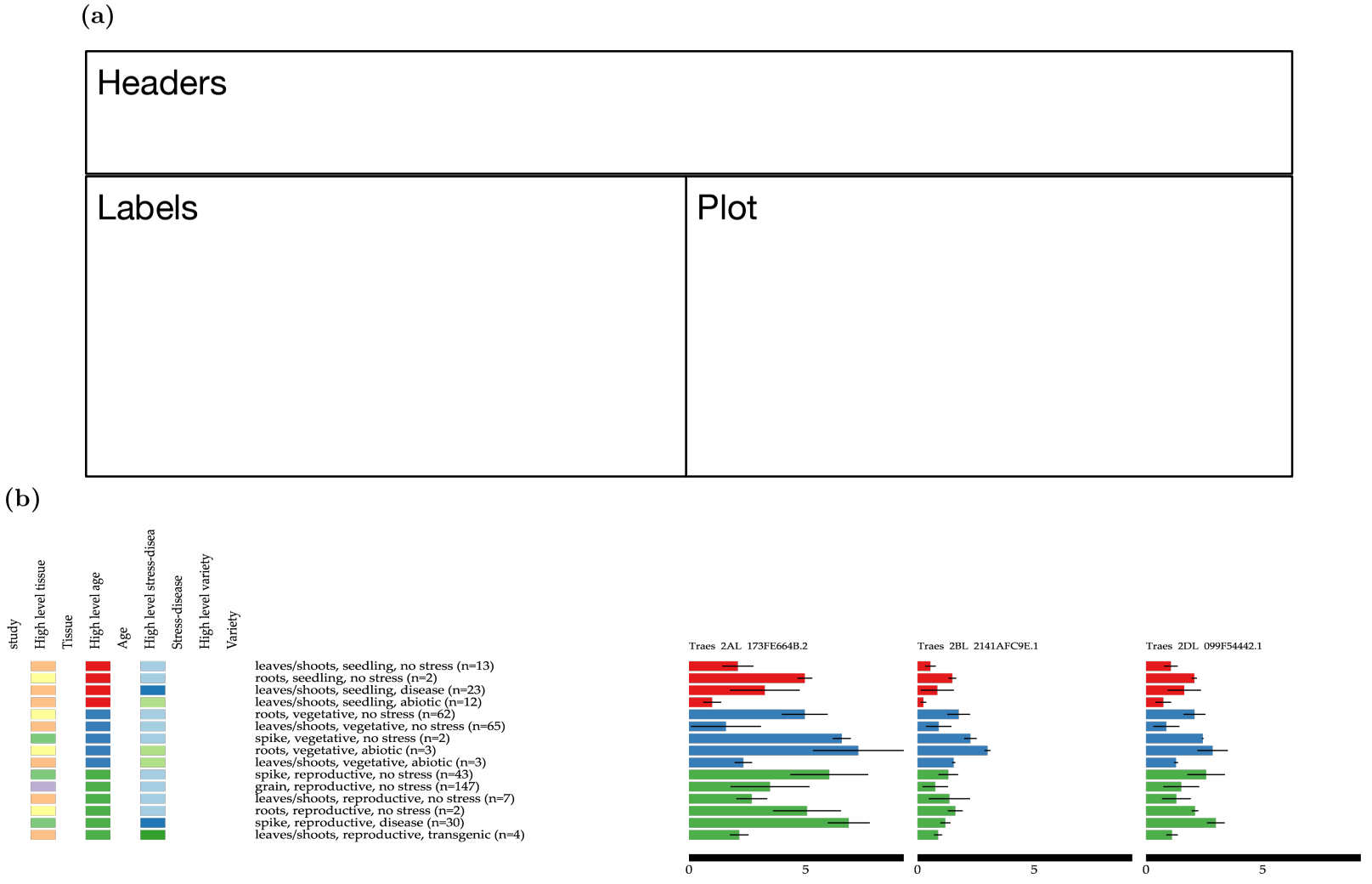


Figure 4.10: expVIP export image. (a) GUI components components exported in image (b). The exported components from Figure 4.9)

.

8. **Homoeologues**: this button displays the graphs of known homoeologues of the original primary gene. This gene is highlighted in bold and the homoeologous graphs will be displayed according to A, B, D genome ordering. The scale of all the homoeologues is the same to facilitate the comparison between them.

9. **Gene names**: each displayed gene is labelled on this block. If the list of genes is too long, the gene names are rotated for readability. In case that a gene name is too long to fit, the font is scaled to the largest size that will fit in the designated area.

10. **Expression level**: the expression level adjusts according to the expression of each set of gene homoeologues. The scale remains consistent across homoeologues to allow comparison. The values are based on the unit selected in the expression unit box (see point 5 above).

11. **Filter**: opens a pop-up window revealing the levels of the selected category. By default, all the levels are selected, but the users can decide to exclude from the visualisation experiments containing any level. The order of the levels can be modified by dragging the levels on the the pop-up window.

12. **Display/hide category**: Each category can be displayed or hidden by pressing the +/− button. As categories are added or removed the expression graphs show the new values for the new groups. Data is not removed when changing the displayed categories, instead the values are distributed according to the new groups (the number of samples remains the same). The colours below the category correspond to each level, and the plot is coloured according to the sorting category.

13. **Expression bars**: These bars represent the expression level for the `n` grouped samples according to the chosen categories (11 and 12 above). When hovering over the bar with the mouse a small tooltip will appear, containing the expression level (tpm or counts) and the standard error (sem) used for the error bars (see 14)

14. **Error bars**: Standard error of the means for the `n` expression values on which the bar graph is based.

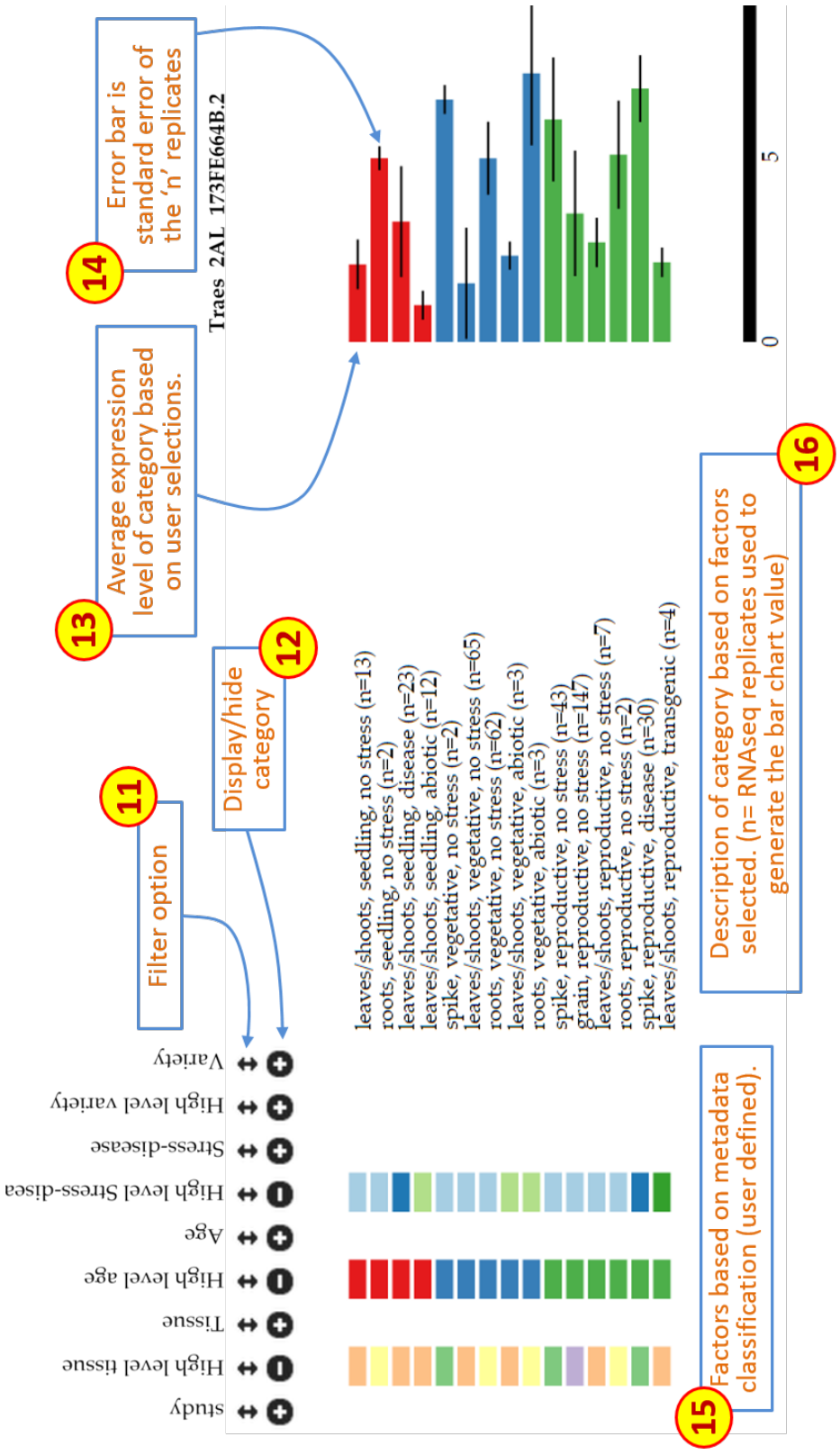


Figure 4.11: Features on expVIP (continued)

15. **Factors**: The colour of the rectangles represents the displayed categories, according to the selection criteria (11 and 12 above). To clarify the meaning of the colour, hovering above the rectangle displays a tooltip with the long name of the examined level.

16. **Description**: Textual description of the grouped factors, according to the selection criteria (11 and 12 above). The number of grouped samples is also displayed.

17. **Expression unit**: For heatmaps, the default unit log2(tpm) as the logarithmic scale provides a better context for comparisons across several genes.

18. **Heatmap**: To compare several genes, the values are represented as a heatmap. The sorting and filtering is done with the same controls as for single genes. Up to 50 genes are displayed in the heatmap, as more genes will degrade the performance of the database, and the visual clutter makes the plot hard to interpret. This view allows to visualise several candidate genes for a trait expressed under certain conditions and quickly asses which one is a good candidate for further research.

19. **Scale**: The scale is calculated according to the highest displayed value in the current heatmap. Since logarithmic values below 1 result in negative values and anything with a TPM under 2 is considered as very low expressed, every value lower than 1 is plotted as 0.

For a comprehensive user manual see Appendix C.

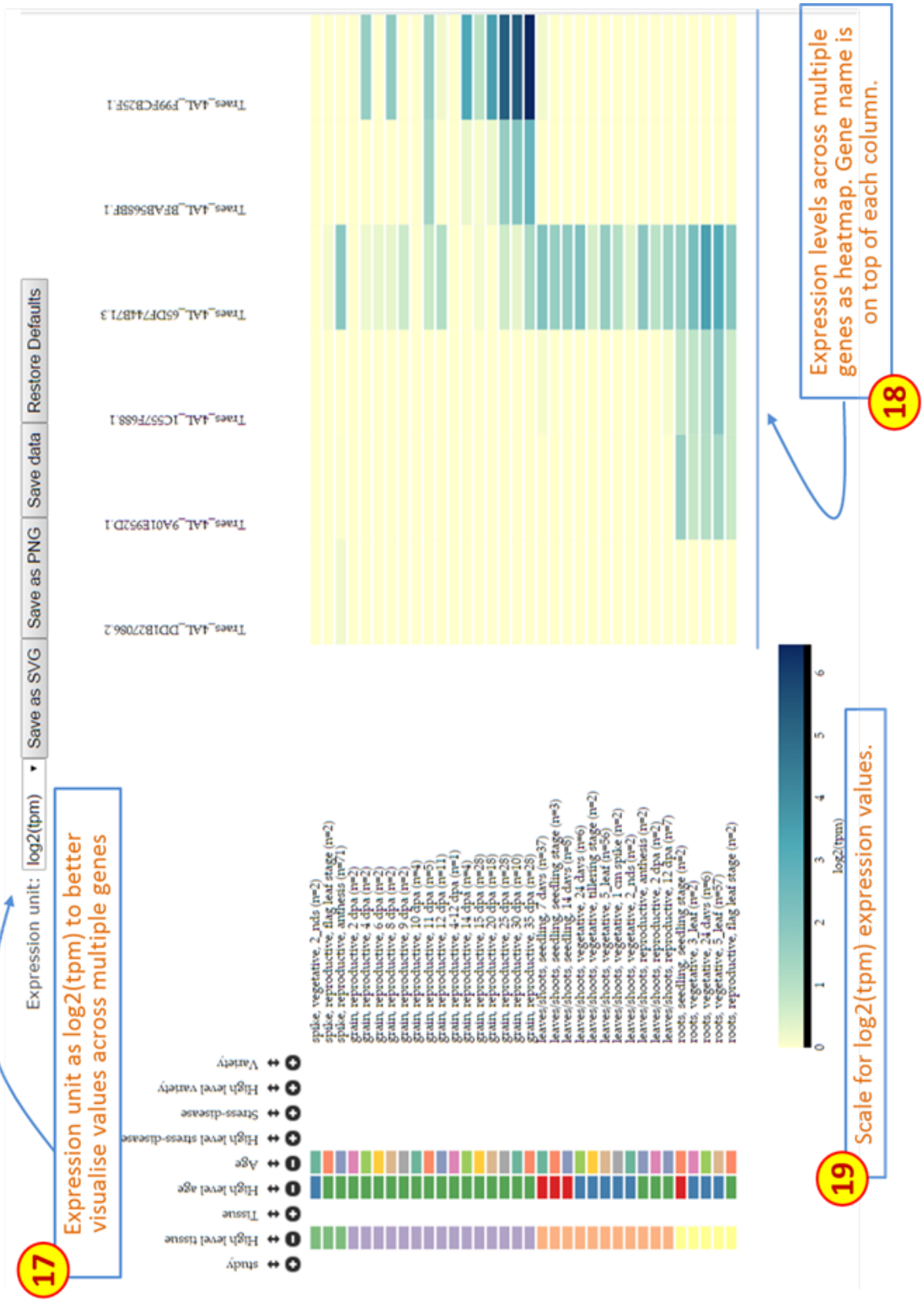


Figure 4.12: Features on expVIP for Multiple gene comparisons

Listing 4.8: Export data example, corresponding to the data plot in Figure 4.9

```
High level age  seedling, vegetative, reproductive,
High level stress-disease no stress, disease, abiotic, transgenic,
High level tissue spike, grain, leaves/shoots, roots,
High level variety  Chinese Spring, other, Nullitetra Chinese Spring,
  tpm SEM tpm SEM tpm SEM
  Traes_2AL_173FE664B.2 Traes_2AL_173FE664B.2 Traes_2BL_2141AFC9E.1 Traes_2BL_2141AFC9E.1 Traes_2DL_099F54442.1 Traes_2DL_099F54442.1
roots, vegetative, no stress(n=62)  4.96  0.99  1.77  0.49  2.08  0.47
roots, vegetative, abiotic(n=3) 7.26  1.95  3.00  0.13  2.85  0.66
leaves/shoots, vegetative, no stress(n=65)  1.59  1.50  0.91  0.55  0.87  0.56
leaves/shoots, vegetative, abiotic(n=3) 2.33  0.38  1.55  0.07  1.29  0.09
spike, reproductive, disease(n=30)  6.85  0.90  1.19  0.22  2.99  0.38
spike, reproductive, no stress(n=43)  6.01  1.67  1.32  0.43  2.58  0.81
grain, reproductive, no stress(n=147) 3.47  1.69  0.76  0.55  1.51  0.77
leaves/shoots, reproductive, transgenic(n=4)  2.15  0.40  0.88  0.18  1.11  0.25
leaves/shoots, reproductive, no stress(n=7) 2.69  0.67  1.37  0.89  1.30  0.62
leaves/shoots, seedling, disease(n=23)  3.25  1.50  0.85  0.71  1.64  0.72
leaves/shoots, seedling, no stress(n=13)  2.10  0.67  0.55  0.23  1.07  0.29
leaves/shoots, seedling, abiotic(n=12)  0.99  0.39  0.25  0.12  0.74  0.34
roots, seedling, no stress(n=2) 4.96  0.32  1.49  0.17  2.07  0.11
roots, reproductive, no stress(n=2) 5.06  1.46  1.62  0.32  2.10  0.14
spike, vegetative, no stress(n=2) 6.55  0.39  2.27  0.27  2.43  0.03
```

4.6 Discussion.

4.6.1 Expression databases in wheat.

In model organisms there are several on-line resources that aggregate the raw data and meta analysis of several expression studies. For example, the Expression Atlas, from European Bioinformatics Institute (EBI), includes over 2,000 studies for *Arabidopsis thaliana* inclusive of other technologies besides RNA-Seq (ie Affymetrix expression arrays). For the purpose of the discussion I will refer to www.wheat-expression.com as expVIP as this is the wheat implementation of the pipeline. However, when I started the development of expVIP the Expression Atlas only included a couple of baseline experiments for wheat and WheatExp had not been published yet (Figure 4.13).

WheatExp was developed roughly at the same time of expVIP and contains 6 RNA-Seq studies (against 16 on expVIP; Pearce et al. 2015b). Four of the studies in WheatExp are on hexaploid wheat and are included in expVIP as well. In WheatExp, the expression for each gene is displayed on the context of the original experiment, making it difficult to compare across studies. However, because the studies are kept independently the factors of each study are dependant on the study. WheatExp allows to search genes by sequence, through a BLAST interface. This feature is not yet implemented in expVIP but is a logical extension which will be set up similarly to the work I've done on the `www.wheat-tilling.com` blast search.

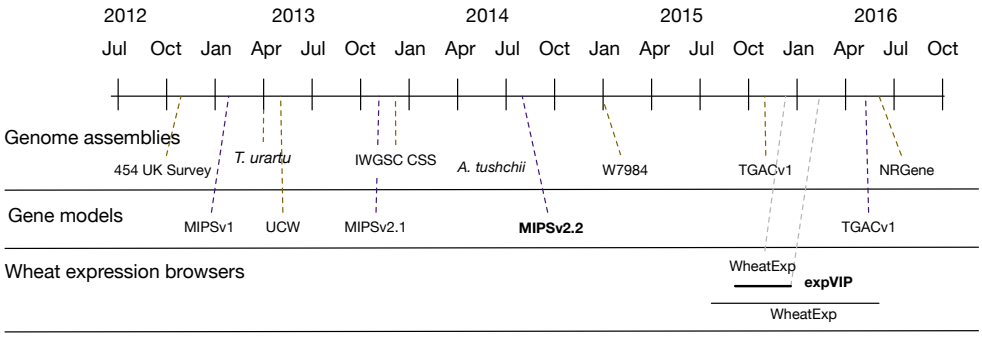


Figure 4.13: expVIP resources time line. In bold the line of the time for development of expVIP and the annotation used. The Expression Atlas has a line starting on the initial study deposited on it till the last update, as of September 2016.

The Expression Atlas from EBI, as of September 2016, contains 10 studies, 4 baseline and 6 for differential expression (Petryszak et al., 2016). ExpVIP contains studies released before EBI started to upload expression experiments for wheat. Even if some of the most recent studies in the Expression Atlas are not included in expVIP yet, we are in the process of updating our tool to include studies published after the initial release. In terms of visualisation, the EBI Expression Atlas includes a heatmap to compare to different factors at the same time for given gene (ie. tissue vs stress); the same information can be displayed on expVIP by sorting by two factors. The Expression Atlas currently uses a similar gene set build as that implemented for wheat through expVIP (IWGSC gene models). However, expVIP provides additional flexibility as it allows users to update the different parameters. For example using the Virtual Machine (detailed below) users can implement the pipeline using a modified or extended gene build. Likewise we have implemented expVIP for the recently released TGACv1 gene models which are now the community standard and available through Ensembl Plants. We plan to allow users to search for the original IWGSC CSS gene models or the new TGACv1 models using a drop down menu. When the final gene models are developed based on the NRGene assmebly a similar approach will be pursued. Retaining all three datasets (IWGSC, TGACv1, NRGene) will allow the community to have a smooth transition as data can be queried based on the different gene model names. This flexibility is not available in WheatExp nor in Expression Atlas.

4.6.2 Quantification of RNA-seq reads.

Most of the RNA-Seq studies report their results in terms of Reads Per Kilobase of transcript per Million of mapped reads (RPKM). This normalisation, which is computed for each feature g in the reference G, requires the count of the number of reads r_g, the feature length fl_g and the total number of mapped reads R (Figure 4.14; equations 4.1 and 4.2 Mortazavi et al. 2008). As the denominator of 4.2 is based on the number of mapped reads rather than the number of nucleotides covered, RPKM does not allow to compare correctly results obtained between different samples, or even results from the same sample when the average read length changes due to variations in the experimental protocol (Wagner et al., 2012). This is relevant for example in the case of the data in

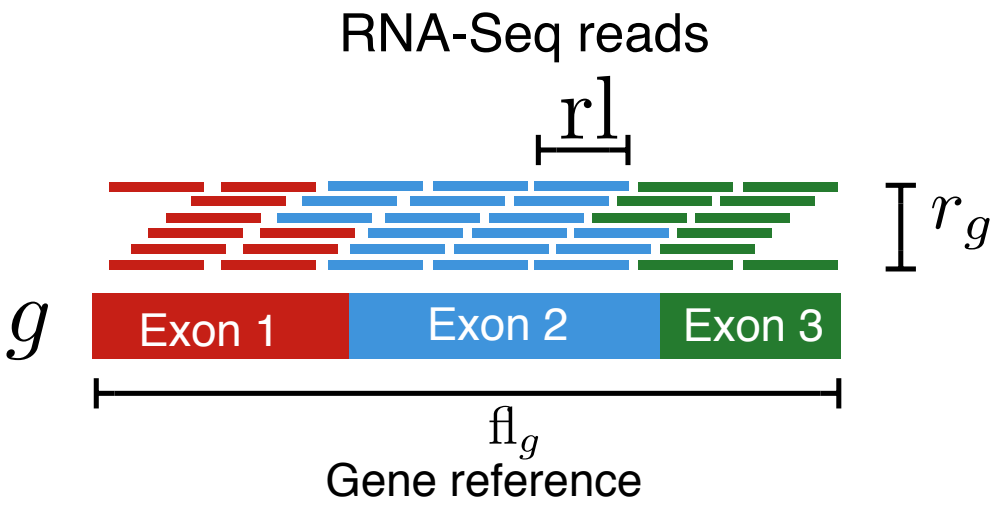


Figure 4.14: Units used for expression normalisation

WheatExp which includes studies with 101 bp paired end data and 50 bp single end data. Using RPKM as is implemented in WheatExp (and EBI Expression Atlas), this data is not treated differently.

$$R = \sum_{g \in G} r_g \tag{4.1}$$

$$\mathrm{RPKM}_g = \frac{r_g \times 10^9}{\mathrm{fl}_g \times R} \tag{4.2}$$

The TPM is an alternative to RPKM that approximates the total number of transcripts T as a normalisation factor (equation 4.3). Besides the previously described parameters, the TPM also includes the read length rl, which is dependent on the study. This formula assumes that each read corresponds to a full observed transcript (equation 4.4; Wagner et al. 2012).

$$T = \sum_{g \in G} \frac{r_g \times \mathrm{rl}}{\mathrm{fl}_g} \tag{4.3}$$

$$\mathrm{TPM}_g = \frac{r_g \times \mathrm{rl} \times 10^6}{\mathrm{fl}_g \times T} \tag{4.4}$$

One of the aims in developing TPM was to be able to compare samples from different studies; as it is more stable across different experiments (Wagner et al., 2012), we decided to use it as the main unit of comparison in expVIP. After we took the decision of which unit to use, we found a couple of tools, Kallisto (Bray et al., 2016) and Sailfish (Patro et al.,

2014), that could calculate the TPMs directly from mapping the reads to a reference (sometimes referred as pseudo-aligner, but the logic behind is closer to a mapping function than actually aligning), without producing a precise alignment. The main advantage of only doing mapping, without aligning is a significant reduction in both the computational resources and the time needed to analyse a sample.

The traditional pipeline to quantify expression from RNA-Seq consists on the following steps:

1. Index the reference. Only done once, as the same index can be used for all the samples.

2. Align the reads to the reference.

3. Sort the alignment and remove duplicates.

4. Quantify the expression.

This is the prevailing pipeline for expression analysis. In my experience, on a computing cluster it takes between 6 to 8 hours to process each wheat sample on a computing cluster, using multiprocessing and up to 24 GB of RAM (Figure 4.15a). This pipeline is usually implemented by aligning the reads with `BWA` (Li and Durbin, 2009) or `Tophat` (Trapnell et al., 2012) and the quantification is performed with tools like `HTSeq` (Anders et al., 2015) or `cufflinks` (Trapnell et al., 2012).

An advantage of using a mapper is that the quantification pipeline is shorter:

1. Index the reference. Only done once, as the same index can be used for all the samples.

2. Map the reads to the reference and quantify the expression in a single program.

Since mapping does not require the precise alignment of every single base on the read and the output is only the quantification for each gene, as opposed to the alignment of each read, the programs implementing mapping take around 15 minutes to run on a 6 GB RAM computer. This amount of memory is now available on desktop computers, making this analysis more accessible for groups that do not have access to an High Performance Computing (HPC) cluster. RNA-Seq mapping algorithms

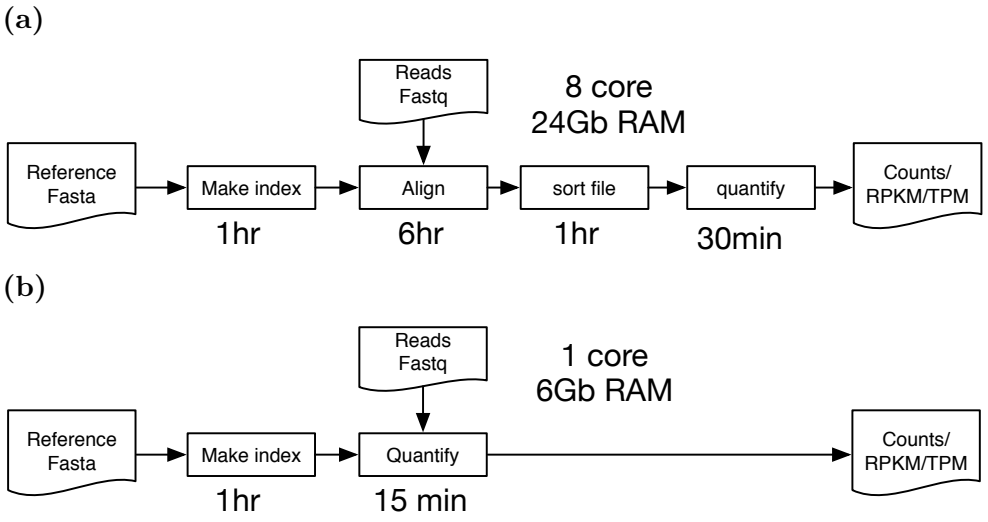


Figure 4.15: Alignment vs mapping pipelines for RNA quantification. (a) Alignment pipeline. (b) Mapping pipeline.

are implemented by `Kallisto` (Bray et al., 2016) and `Sailfish` (Patro et al., 2014).

I decided to integrate `Kallisto` into expVIP because the algorithm is able to walk through different splicing junctions via a T-DBG (see Section 4.1.4; Bray et al. 2016), as opposed to Sailfish, which is based on counting k-mers only (Patro et al., 2014). Since homoeologues with a high level of identity may form a bubble in the graph, in a similar way to small indels or SNPs (Leggett et al., 2013), `Kallisto` should be able to assign the reads to the correct k-compatibility class for their corresponding homoeologue.

4.6.3 Next steps in wheat expVIP.

Some features that could improve expVIP in the long term and that already present in other expression browsers would include searching by sequence, by gene ontology and a heatmap of a particular gene displaying two different factors on each axis . A feature that expVIP could leverage from other genomic resources is the retrieval of genes flanking a gene of interest, or between genetic markers. The upcoming assemblies , from the IWGSC (Clark, 2016) and TGACv1 (Pozniak, 2016) with longer scaffolds in conjunction with the high resolution genetic maps from Wang et al. (2014) and Chapman et al. (2015) can enable such kind of queries (resources described in Section 1.4). Likewise, once the NRGene sequence

is annotated, it will be possible to generate this information which will be extremely useful to refine candidate genes for QTL or in positional cloning projects.

For the TGACv1 assembly, an improved wheat annotation was made available after the initial release of expVIP (Figure 4.13). The International Wheat Genome Sequencing Consortium (IWGSC) is currently working on developing an updated annotation based on the new NRGene assembly. In the near future, expVIP will be updated to include those annotations and some development will be needed to allow the comparison between annotations, at least while the community adopts a canonical reference.

4.6.4 Comparisons within and between species.

The same mechanisms to compare expression between different references can be used to compare the expression between different organisms. However, the current implementation uses the homology table with one column for each genome group in hexaploid wheat (A, B, and D; Figure 4.16a). To be able to allow the inclusion of different polyploids with different genome names, to compare homologoues and paralogues effectively and to add any arbitrary relationship between genes, the homoeologues table needs to be updated. Instead of representing triplets, the table should contain binary relationships; each gene pair will have a type to be able to distinguish between relationships. Furthermore, each gene set should be linked to a species. With that explicit relationship, equivalent genes from the same species, but coming from different gene models can be identified. Likewise, genes known to be conserved across relatives (ie Barley vs Wheat genes) can be compared through these modifications to the database (Figure 4.16b).

To the best of my knowledge, none of the expression browsers available for wheat, or other polyploid species, allow the direct comparison between homoeologues. However, the effect of different related genes is a topic of active research in polyploids. Making the relationship of the expression between related genes easily accessible can provide some initial evidence of having an uniform expression across homoeologues or of a triplet with a dominant gene. Likewise, when the update to the table that keeps arbitrary relationships between genes is completed, it will be possible to find if related genes have a conserved expression pattern across

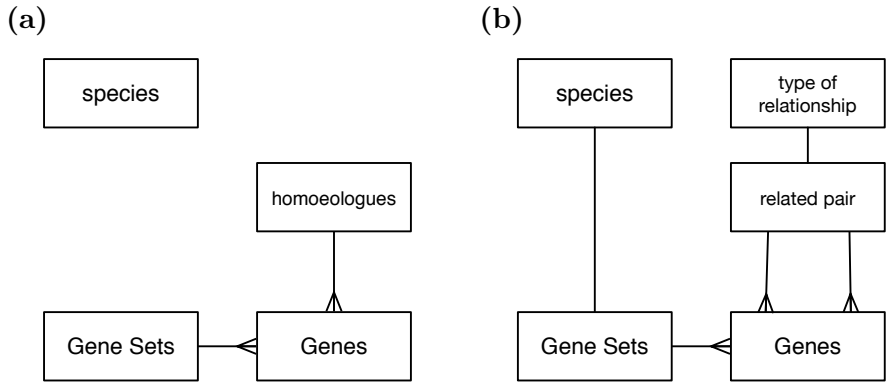


Figure 4.16: Changes to improve gene comparisons. (a) Current implementation of homoeology. (b) Proposed implementation to extend the types of relationships between genes.

species. To achieve this though it will be critical that the metadata uses common nomenclature across species. For example, proper plant ontology (Cooper et al., 2013) terms should be implemented in the metadata to be able to conclude that expression patterns are conserved across the same tissue and developmental stage in different species. This is not a trivial undertaking but a new PhD project at Earlham Institute (EI) is looking in ways to facilitate this feature.

4.6.5 An open access resource.

Since its conception, I wanted expVIP to be open source and available to the community. As part of the project, I released the visualisation component as a BioJS component (`bio-vis-expression-bar`; `http://biojs.io/d/bio-vis-expression-bar`; Yachdav et al. 2015). In a collaboration with the Earlham Institute and eLife, another PhD student is working to integrate the visualisation plugin as a live figure. If I had kept the code closed, this potential use of the component would have never happened.

The webserver is also open and hosted on github (`https://github.com/homonecloco/expvip-web`), with a tutorial on how to load the data on a personal server (Appendix C). As the quantification tool used by expVIP has a relatively low memory requirement, I was able to prepare and preconfigure two virtual machines, one without any data loaded and one with all the data used in the original article.This allows the comparison of private projects in the context of previous studies. On the Norwich Research Park, at least two groups are actively on using expVIP to make

their data available to the community on other species. We had also been contacted by both the team behind the annotation of TGACv1 and the IWGSC NRGene assembly to include the upcoming annotations to the public instance of expVIP.

The public server at `http://www.wheat-expression.com` has received visitors from the majority of the wheat research institutes around the globe (Figure 4.17). Since March 2016, when the Google analytics tracker was setup in the website, over 1,921 users have visited the site. Most of the users are based in the UK (34%), and within the nation the majority of the traffic originates from Norwich, Cambridge, Harpenden, London and Dundee. Those cities have research institutes that work on wheat, so it is very likely that the users are real. Most of the international users are coming from the US, China, Australia, India, Germany and Canada. In those countries, most of the visitors also come from cities where wheat institutes are based (i.e. Perth, Ludhiana, Quedlinburg (IPK), etc). Furthermore, around half of the sessions stay on the website to access either the the individual genes or the heatmap with an average session duration of over 4:30 across the >3,500 sessions. Around 25% of the sessions consult their genes using the heatmap, suggesting that they have a list of candidate genes for a trait of interest to select by comparing their expression.

Overall, expVIP has met its aims (Section 4.1.2). I designed a relational database capable of storing several RNA-Seq experiments with their corresponding metadata. The expression analysis has been automated, to facilitate the process of running Kallisto, the selected quantification tool. The stored data can be visualised to compare the expression across several conditions, and the visualisation tool allows the arbitrary grouping and selection of studies. Finally, the open source code and the virtual machines facilitate the adoption of expVIP on other communities, not necessarily working on wheat.

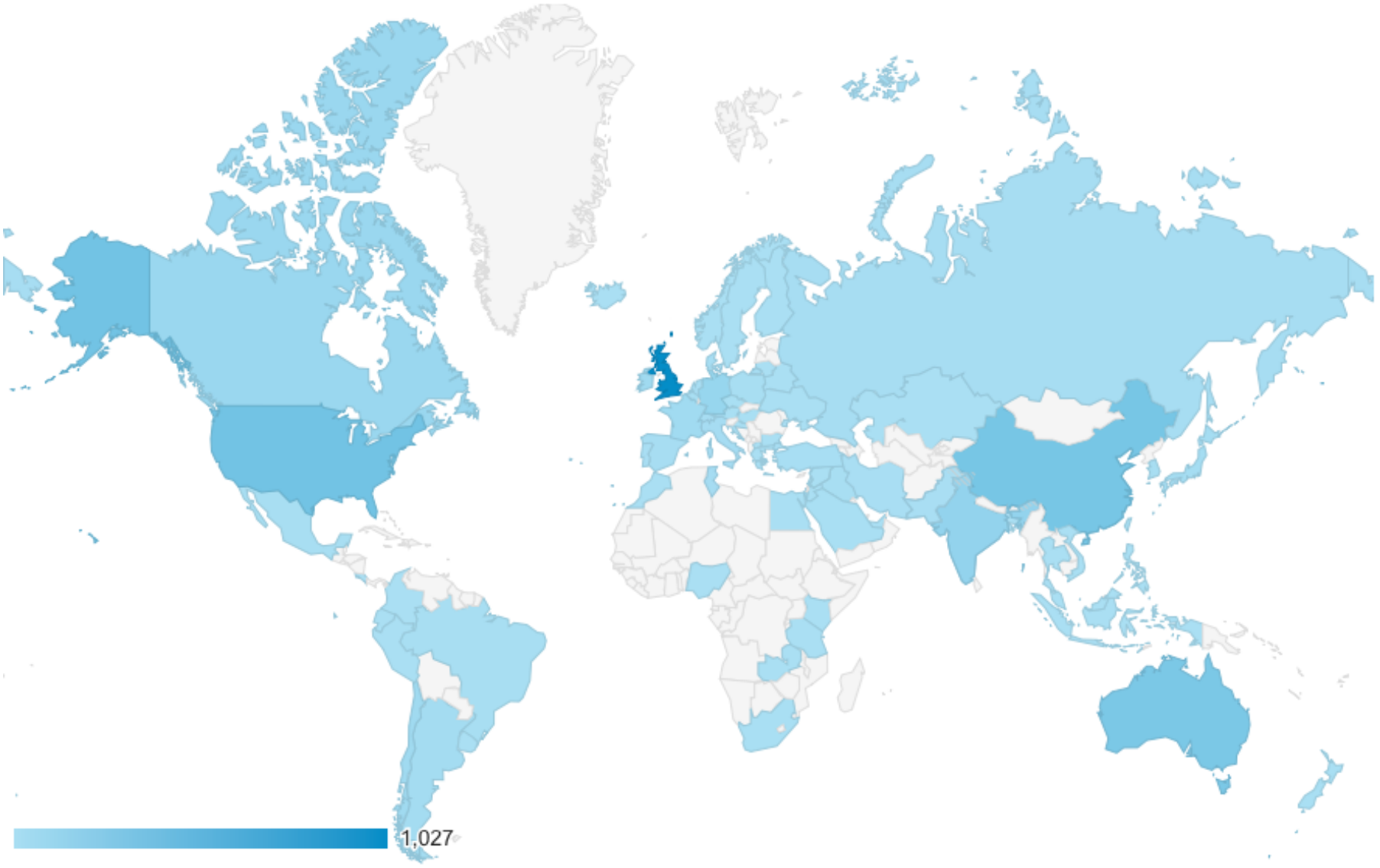


Figure 4.17: Heatmap of the number of unique sessions of expVIP in the world. The shade of blue represents the count of sessions per country. The highest number of sessions (1,027) come from the U.K.

Chapter 5

General discussion and final remarks

Knowledge from computer science can be applied to produce software for specific needs, and which can also be useful for the community. One of the limitations though is that most approaches are developed for diploid systems and are sometimes not compatible with polyploid species, such as wheat. Polyploidy adds an extra level of complexity (due to homoeologs) and in the case of wheat the large genome size also hinders certain approaches to genome analyses. Therefore bespoke tools are required to deal with these barriers.

In this thesis we have taken advantage of technological developments and in genomic resources to generate a series of solutions to several of the problems associated with polyploidy. These methods and approaches are not restricted to wheat, but can be applied and implemented to other polyploid systems. Diploid species with recent whole genome duplication events (such as *Brassica rapa* and *B. oleracea*; Cheng et al. 2014) present similar issues surrounding closely related gene copies and the methods and approaches developed here are likewise applicable.

I have discussed individual the elements of project at the end of each chapter. However, looking forward my interest is to integrate this data into a single scheme. I outline this in Figure 5.1 and discuss it below.

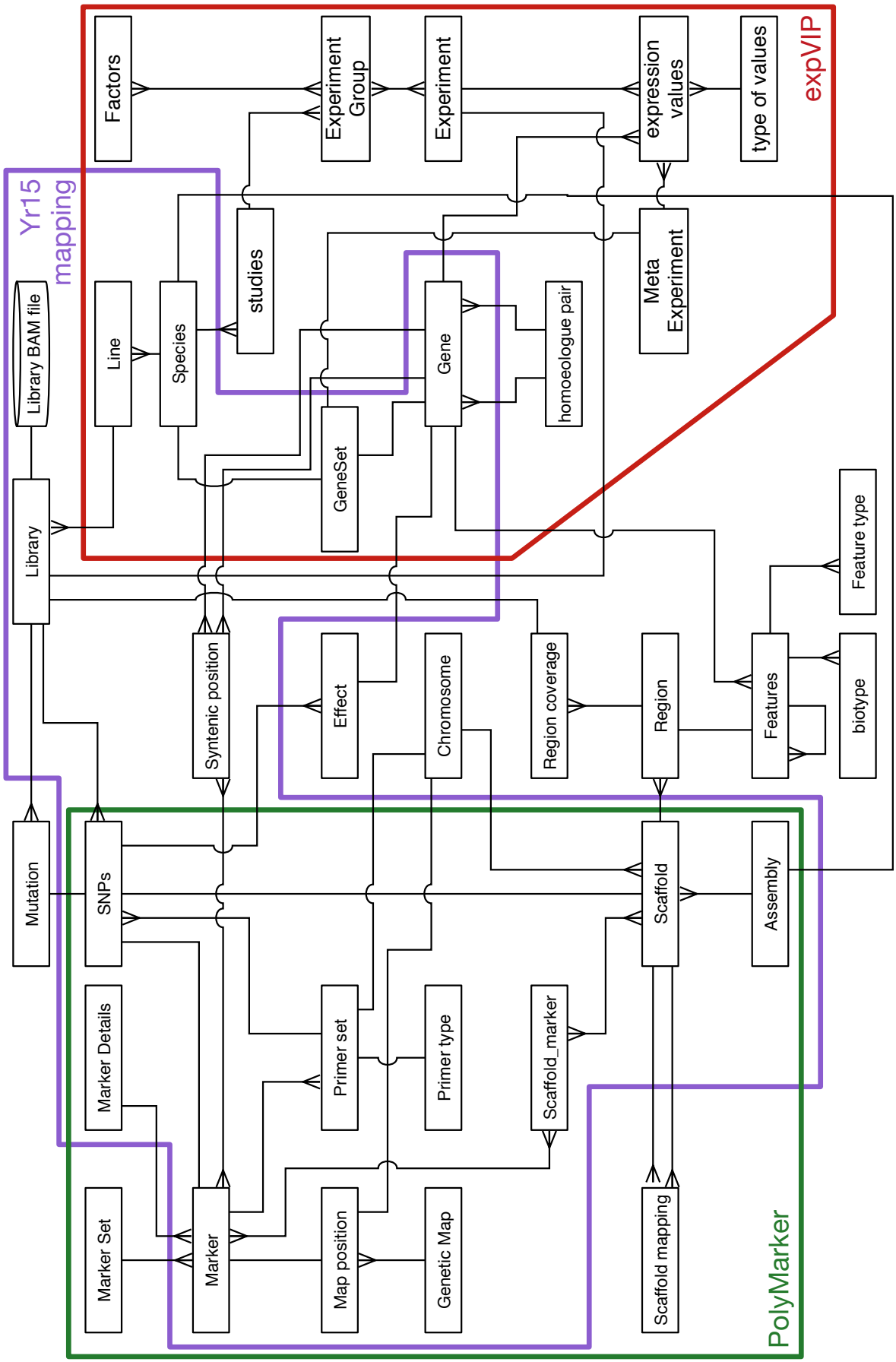


Figure 5.1: Relational database integrating all the datasets. The boxes represent the tables that contain information related to each chapter.

5.1 Integration of different genetic maps

Genetic maps are a common starting point to search for a locus linked to a trait, both for breeders and academic researchers. For example, in Chapter 3 previous genetics maps had already identified the short arm of chromosome 1B as the locus for *Yr15* (Murphy et al., 2009). Furthermore, I was able to confirm an enrichment of SNPs linked in the expected region thanks to the mapping of the markers included in the genetic map from Wang et al. (2014) to the CSS scaffolds (Mayer et al., 2014). Since my initial analysis, other genetic maps with a higher resolution have been published (Chapman et al., 2015; Allen et al., 2016; Winfield et al., 2016) and these could be further integrated in future analyses. The relationship between the tables used in the genetic map is shown in Figure 5.2

Genetic maps are produced from the genotype of a population with several markers (marker sets). Those markers can be developed explicitly for the genetic map or from an already published marker set, usually in the form of an SNP array. A database containing several genetic maps should be able to distinguish the origin of the used markers. However, a genetic map may contain markers from several marker sets as well. For those reasons, a genetic map is not connected directly to a marker set, but the relationship is maintained through the markers and their position in the genetic map. For example in Simmonds et al. (2014), the genetic map across a target locus includes microsatellite, diversity array technology (DArT), CAPS, and SNP array derived KASP markers.

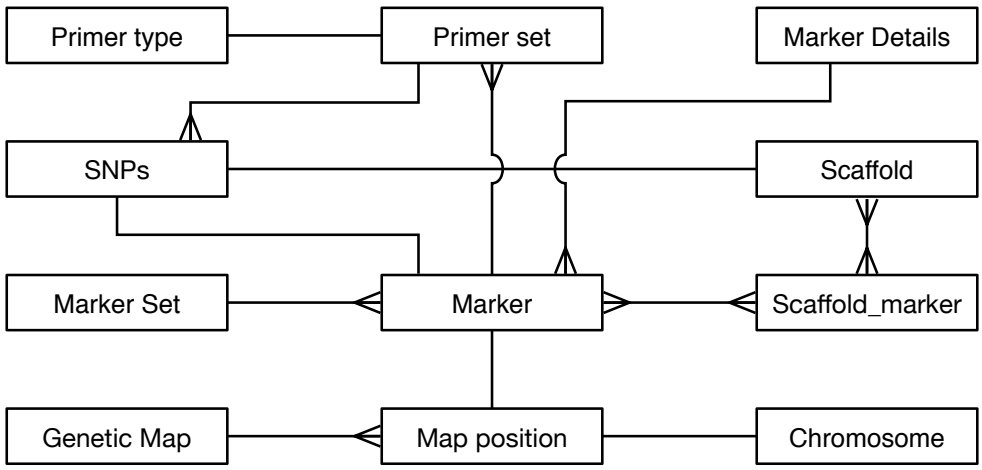


Figure 5.2: Tables to store information about genetic maps.

To be able to use the genetic markers in a genomic context, the sequence of the markers must be mapped to a reference. The assembly may be in a single pseudomolecule (such as the new NRGene assembly) molecule or be separated in scaffolds (such as the IWGSC CSS or TGACv1 assemblies). If the assembly is fragmented, the map can be used to anchor the scaffolds to a genetic position. If the assembly consist of long scaffolds the genetic map and the positions can be used to find if the lines used for the map have a rearrangement event. If a rearrangement is present, the collinearity between the genomic reference and the genetic map is not conserved. Having the markers and scaffolds in the database simplifies this kind of analysis, as all the needed data is readily available. This will prove important as new genome references become available for a diverse set of wheat varieties as well as high density maps for large mapping populations. For example, Earlham Institute (EI) are currently re-sequencing Avalon and Cadenza, two important lines in many UK variety pedigrees. A large Doubled Haploid (DH) population from these lines is available and several high density genetic maps have been made from different Single Nucleotide Polymorphism (SNP) arrays (Winfield et al., 2016; Allen et al., 2016). It will be fascinating to see how the integration of these high density maps and the long range scaffolds produced by the EI genomic assemblies will help elucidate local rearrangements which are proposed in this population (Allen et al., 2016).

Furthermore, PolyMarker (Chapter 2) has been used to design KASP assays for most of the primers in the 90k (Wang et al., 2014) and 820k Winfield et al. (2016) SNP arrays. The primers for the assays can be integrated in the database. This allow a use case were known flanking markers are queried and the database can return a list of possible markers with the primers already designed to be validated on a mapping population. This is now routinely done in many labs based on personal communications. The general approach has been used in Simmonds et al. (2014) who genotyped NILs with the SNP assays. After identifying polymorphic markers these had to be manually converted into KASP assays; the delay in designing primers in this study inspired in part the development of PolyMarker. Similarly, many labs routinely use RNA-Seq for SNP discovery and spend several weeks or months in primer design (Shatalina et al., 2013). Discussions with this group and others also supported the need to rapidly convert *in silico* SNP data into functional

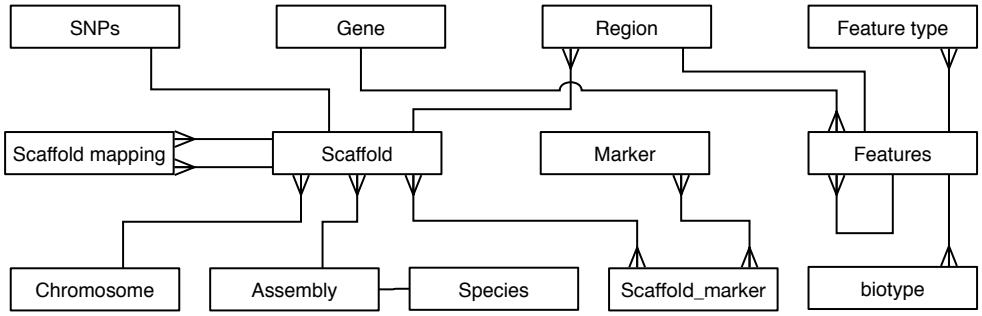


Figure 5.3: Tables to store information about genome assemblies and their annotation.

individual SNP assays. PolyMarker has largely succeeded in removing this important bottleneck.

5.2 Integration of different genome references.

The efforts to produce a wheat reference genome had been focused on the Chinese Spring (CS) landrace. CS is only cultivated as a research line, as it is susceptible to several pathogens and its yield is inferior to modern varieties. The reason for CS to be the selected cultivar to be sequenced as reference is historic: it has long been a variety for research. CS was originally chosen because it was able to cross with rye. It has also been used to produce lines with chromosomic aberrations, useful to find if any particular chromosome is responsible for certain traits (Sears and Miller, 1985). Figure 5.3 includes the tables used to store assemblies, the relationships between them and their annotation.

New assemblies are required to address the shortcomings of the use of CS as a genome reference and to include the diversity from other lines (Allen et al., 2016; Winfield et al., 2016). The assemblies may include their own annotation, and that should be reflected in the database too.

As of September 2016 there are four sets of genomic sequence used by the wheat community:

1. A 454 whole genome shotgun sequence that is unassembled, and each read is treated as a scaffold (Brenchley et al., 2012).

2. The genome assembly and annotation from the Chinese Spring Chromosome arm survey sequence (CSS) done by the IWGSC (Mayer et al., 2014).

3. A whole genome shotgun sequence from a synthetic wheat, without a corresponding annotation (Chapman et al., 2015).

4. The whole genome shotgun sequencing and annotation from CS (TGACv1; Clark 2016).

All those references can be aligned to each other to find corresponding regions. The corresponding regions can be stored in the scaffold mapping table. Furthermore, the scaffolds can be mapped to related species, such as *Brachypodium distachyon*, *Oryza sativa*, *Sorghum bicolor*, and *Hordeum vulgare* to find syntenic blocks.

Each genome assembly is usually annotated with their genes and other features. To include the annotation, each scaffold can contain several features. As some features consist on sub-features, like genes conformed by several exons, a recursive relationship is included in the features tables. With the support of the scaffold mapping, the different annotations can be projected on different references. Gene models, like the ones described in (Krasileva et al., 2013), and genetic markers can be aligned to any of the reference genomes.

With all those relationships available, the *In silico* mapping in Chapter 3 could be produced on several references at once. Also, the relationship between different gene models could be simplified, as the corresponding features will share coordinates. Likewise, the co-expression of genes located in the same region is an useful feature for expVIP (Chapter 4).

5.3 Integration of expression data and beyond

A database to integrate expression studies was developed for expVIP (Chapter 4). I designed the database to be as extensible as possible, already with the idea of having an integrated database for different types of resources for wheat. To better unify expVIP with the rest of the database, the experiments can be assigned to a particular library. In that way, when the RNA-Seq experiment is used for SNP calling, a relationship for it will be available. A more comprehensive discussion of the tables in Figure 5.4 is found in Chapter 4.

On Chapter 3, I described how RNA-Seq can be used as a reduced representation method to call for SNPs. However, RNA-Seq is primarily used to analyse differential expression, which can be used to find candidate genes involved in a trait. Other studies have shown that it is possible to do differential expression and SNP calling to improve the candidate genes linked to a trait (Lopez-Maestre et al., 2016). On the current design of the database it is possible to integrate both types of analyses (Figure 5.5). Because the database is able to integrate previously published studies from different source, it is possible to link the expression to variations previously explored.

The effect of the SNPs can be predicted with tools like snpEFF (Cingolani et al., 2012) or Ensembl VEP (McLaren et al., 2016). I have implemented this part of the database for the `http://www.wheat-tilling.com` website, which contains all the mutations, their effects and primers (see Section 2.3.2) described in Krasileva et al. (submitted 2016). Currently the database in the tilling website doesn't include any information regarding the expression. Hence, to see if a mutation is in a gene differen-

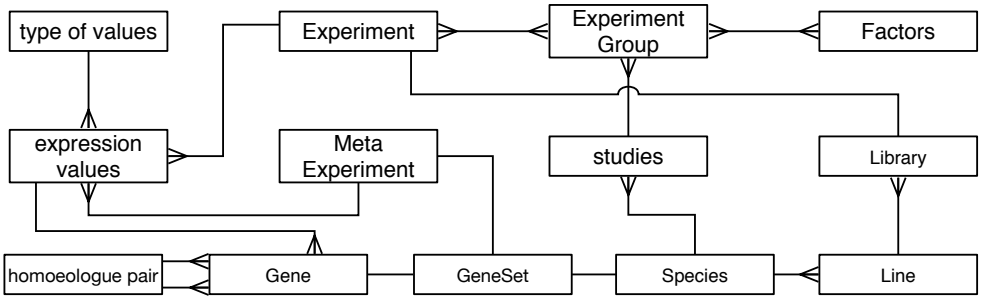


Figure 5.4: Tables to store information about gene expression.

tially expressed requires a separate visit to `www.wheat-expression.com` (Chapter 4). If the two databases were merged as proposed, it would be possible to have a query to find all the SNPs in genes that are differentially express under certain conditions. Moreover, this integration would allow users to query for specific mutations which result in truncations in all differentially expressed genes in a given chromosome interval.

To increase the available information, the SNPs and the corresponding alleles for lines with a known genotype can be added to the database. Several varieties had been genotyped with the 820k (Winfield et al., 2016) and 90k (Wang et al., 2014) SNP arrays, as well as with exome capture methods (Jordan et al., 2015). Despite being publicly available in CerealsDB, the size of the datasets require specialised bioinformatic knowledge, as regular spreadsheet software is unable to load all the information and stay responsive. For that reason, a database to query this datasets would be valuable by itself. However, integrating the marker information on the general database can be useful to validate if SNPs called for a studied line are consistent with the expected genotype. This kind of validation of the variety of the sample has been done with custom scripts in (Hubbard et al., 2015). In addition the integration of SNP data across the more contiguous physical sequence now available allows long-range haplotypes to be established.

Finally, the coverage of certain regions can be stored as counts for the expression analysis, or to analyse the copy-number variation. On the same mutant population I have developed an algorithm to detect homozygous deletions from exome capture. Since the sequencing is incomplete, finding the exact location where a long deletion starts is difficult. How-

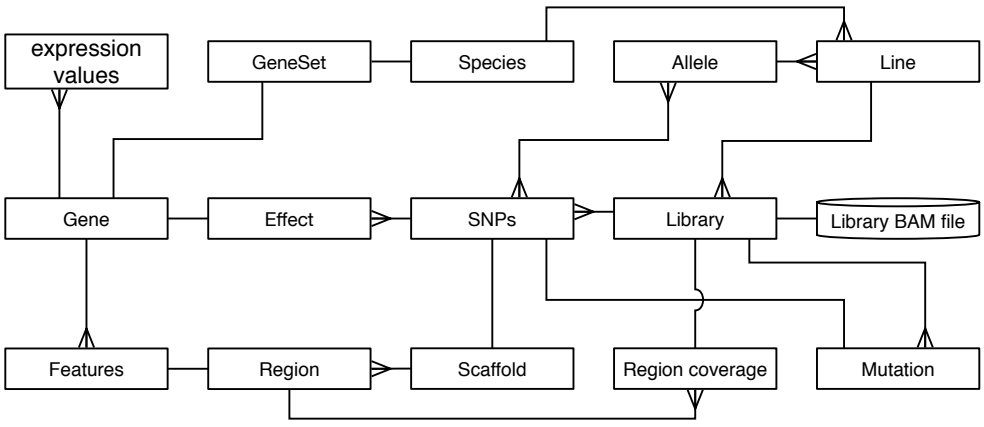


Figure 5.5: Relationship of different types .

ever, the relative coverage of the exon can be used as a proxy for the deletion. We have proved that for homozygous deletions this approach works (PolyInDel; Section 2.4.1; Krasileva et al. submitted 2016). In the future, I would like to extend the algorithm to detect copy number variations. Storing the coverage at the exon level in the database, along with the annotation of the deletions, provide another set of candidate genes if the phenotype of a line is known.

Overall, having a single database with different types of genomic and genetic data, alongside with a reliable annotation can simplify functional genomics.

5.4 Integration of sequencing experiments and genetic maps

Genomic data by itself is a valuable resource that can help to find SNP markers, differential expression under different condition and structural variation of the genome. However, it is not the only available tool for crop scientist and breeders to improve wheat. Genetic maps and their associated markers had been used to identify locus linked to traits and for selective breeding, as described in Chapter 3.

Another source of candidate genes is synteny with relative species (Moore et al., 1995). Both *Brachypodium distachyon* and *Hordeum vulgare* can be used as models for wheat as they are closely related diploid organisms with a small genome size (*Brachypodium*) or a more advanced genome sequence (barley; full genome sequence submitted a few weeks ago). In the case of barley, the seven chromosomes are mostly collinear (Rustenholz et al., 2010) with each of the wheat genomes (A, B and D). To take advantage of this relationship, the wheat genetic maps can be linked to genes in more than one species (Figure 5.6). In personal communication with other members of the Uauy group I became aware that it is possible to test the effect of a candidate gene on a related diploid species, where the effect of a change in a single gene can not be hidden by homoeologues genes, as happens in wheat.

With a candidate gene tested in a related species, the syntenic relationships can be added as supporting evidence for results coming from different datasets. For example, if a gene of unknown function is found to

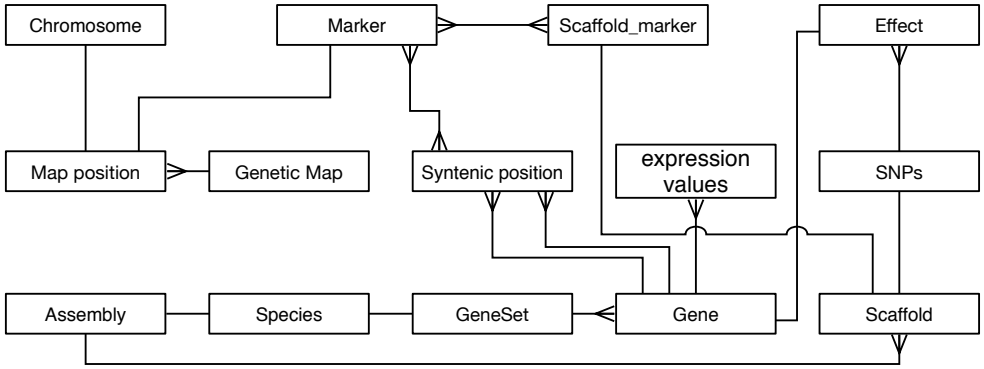


Figure 5.6: Tables to store information about gene expression, SNPs and their effects, assemblies and, genetic maps.

contain a SNP which leads to a premature termination codon, it can be compared to the corresponding gene in barley, which may have a known function. Since the gene is mapped to a scaffold, and the scaffold is located in a genetic map, it is possible to validate if there are any QTLs for the same function observed in barley. This can be logically extended to better characterised species like rice and can be updated with new information on genes as this becomes available.

5.5 Integration with other services

Currently, the publicly available wheat resources are scattered as supplemental materials on their corresponding publication or available as *ad hoc* systems focused on a particular field. For example Ensembl has two different views for every organism: from the genomic point of view and from the expression data. The Collaborative Open Plant Omics (COPO; Davey et al. 2015) is trying to integrate different sources and types of data by connecting the data providers. This approach requires the cooperation of the service providers, which have their own view of what is important. I believe that in order to effectively integrate the resources it is necessary to understand how the users are likely to interact with the data.

In order to increase the exposure of PolyMarker (Chapter 2) and expression Visualisation and Integration Platform (expVIP) (Chapter 4), an integration with other online resources is necessary. So far, expVIP links to Ensembl to get the details of a displayed gene. However, there is no link back from a gene in Ensembl to expVIP. To address this it

should be possible to contact Ensembl to link back, but it may require a custom interface between both sites. This requires an active effort from all the service providers. Initiatives like COPO try to reduce the friction between data providers, by providing a common language between sites. In the future, the proposed database and the webservices described earlier should implement an interface to communicate with other databases.

5.6 Final remarks

I started my PhD with a strong computational background, but with a limited knowledge of wheat genetics and breeding. When I was looking for a PhD I wanted an interdisciplinary supervisory team, with a member with bioinformatic background on one side, an one with a strong knowledge of biology. Since I had previous experience with processing sequencing data, the project led by Mario Caccamo and Cristobal Uauy on using sequencing to improve wheat caught my attention.

On the first year of my PhD I did what at the time seemed as regular bioinformatic analysis: aligning sequences and calling for SNPs. However, as the project of the mapping of *Yr15* progressed I found out more about genetics and how it is impossible to interpret sequencing data without understanding the context surrounding the experiment (Chapter 3). As I got more involved in the genetic mapping project I took the opportunity to go and work in the lab to complement my bioinformatic abilities. I was able to run the KASP assays I had designed and develop the genetic maps for my publication. I also confirmed that the wet lab is not my vocation, but I can better appreciate the work behind it. Also, I found out that the level of uncertainty when doing the experiments in the wet lab is higher than doing the computational analysis and the importance of generating high-quality data interpretation before initiating wet-lab work.

While working on the wet lab I figured out that some of the common analysis can be automated, like the primer design. When I faced the prospect of designing manually 50 primers to validate the SNPs and generate a genetic map for *Yr15* I thought that it is the kind of job that computers can do faster with less mistakes. This led me to take the initiative to make an automated pipeline to design primers in polyploid organisms (PolyMarker, Chapter 2). To properly design PolyMarker I had to un-

derstand the relationship between the three genomes of wheat and the caveats of working with the CSS assembly, which I helped to assemble before starting my PhD. That also changed my vision of bioinformatics. I figured out that it is not necessary to have the perfect reference, or the most sound method, if the resource you are producing is not accessible to the target community.

It was around that time that I noticed that the number of available resources for wheat was growing faster than the community could learn how to use them effectively. For example, the development of a massive number of markers can be used to produce high resolution genetic maps, with the right population. However, the size of the tables represent a problem to excel, one of the common tools used to organise tables. Having a computational background I thought that the kind of queries that could be done with those files could be implemented as a relational database. At that point the CSS scaffolds were adopted by the community, despite being relatively short, they enabled analyses that were impossible before. I figured out that the high resolution maps could be used to give some order to the scaffolds, and part of me wanted to make an improved version of the scaffolding tool I worked on for the pig genome (Groenen et al., 2012). However, the assembly didn't have enough resolution to produce a proper pseudo-molecule and other efforts were being carried out to improve the assembly.

Nevertheless, the idea of integrating different types of data matured enough as to start writing a prototype of the database. When discussing with the group how to make available the public RNA-Seq samples deposited by the community I believed that I could leverage on part of my design to power an expression browser. Originally, we thought on looking for other open source expression browsers, but none of them had the flexibility required to work on polyploids and they were designed with model organisms in mind, with stable resources and conventions followed by the community. So I decided to write the the expression browser that eventually became expVIP (Chapter 4). For the visualisation I leveraged on my previous experience developing BioJS components, as I worked on a component to visualise BAM files in the browser, under the Google Summer of Code in 2014.

During the last year I extended the database design and implementation, as we required to display the data from the tilling population

described in Krasileva et al. (submitted 2016). As the data grew in size and complexity, a single table containing all the mutations with their corresponding effects and primers was not enough. Since I already had been developing a prototype of the database to simplify my own analysis when combining data sources, I thought it would be possible to leverage on that code. However, the tables that would be useful for the database were different to the tables I had developed already. At the end, the combination of the expression and tilling databases, combined with my initial prototype conform the relationships described in the final discussion (this chapter).

After the four years of my PhD I became convinced that in order to produce bioinformatic software that is powerful and usable it is a requirement to understand both the biological processes to solve as well as the computational methods and software development practices to be be implemented.

Appendix A

Supplemental tables

A.1 PolyMarker supplemental tables.

Table A.1: Validation of mutations on M_4 on Cadenza

IWGSC contig	Line	Pos	WT	Mut	Predicted	M_4	Primer 1 (Cadenza)	Primer 2 (mutant)	Common Primer
IWGSC_CSS_3B_scaff_10445294	Cadenza1772	6019	C	T	het	het	caggatAgtGggactgtcaaaG	caggatAgtGggactgtcaaaA	ggagacGGctGtggacatT
IWGSC_CSS_3DL_scaff_6955403	Cadenza1772	2418	C	T	het*	hom	tcagCggattgtcgggatG	tcagCggattgtcgggatA	tgtcCatgaaTcttgtccacG
IWGSC_CSS_4AL_scaff_7106846	Cadenza1772	11277	G	A	hom	hom	tgggatccatgcctacactG	tgggatccatgcctacactA	gatggtGgatttgccgctA
IWGSC_CSS_4AS_scaff_5991335	Cadenza1772	15710	G	A	hom	hom	ctggccctgcgctgctaC	ctggccctgcgctgctaT	gtggaaGttcagaaggaccaG
IWGSC_CSS_4BS_scaff_4956646	Cadenza1772	252	G	A	het*	hom	gcaggttgacttcccggaG	gcaggttgacttcccggaA	tGaggtacgaGcTaaagAaagC
IWGSC_CSS_4DS_scaff_1715962	Cadenza1772	1225	G	A	hom	hom	cagctgtggTatctcaactgG	cagctgtggTatctcaactgA	CcCtGaaACACcGtttggaT
IWGSC_CSS_5AL_scaff_2763407	Cadenza1772	2119	G	A	hom	hom	gcgacGaacctcgagatctG	gcgacGaacctcgagatctA	gaTggcaAtcgtCgtgcA
IWGSC_CSS_5AS_scaff_1548786	Cadenza1772	12625	C	T	het	het	AtaggcacattgctagactgaG	AtaggcacattgctagactgaA	ggattgggtgttgcacgC
IWGSC_CSS_5BL_scaff_10849226	Cadenza1772	2289	C	T	het*	hom	cctgacatcattgttcacgatC	cctgacatcattgttcacgatT	cactccgaggtgtccatgaT
IWGSC_CSS_5BS_scaff_2270737	Cadenza1772	2262	G	A	hom	—	attcCTgtgttgtggCaaatgaG	attcCTgtgttgtggCaaatgaA	taaGcacaaAccctccagctgG
IWGSC_CSS_1AL_scaff_3022915	Cadenza1661	891	C	T	hom	hom	ccacagtgagactcctattgaCG	ccacagtgagactcctattgaCA	atgtctgattcGtcGtagtcC
IWGSC_CSS_1AS_scaff_3297240	Cadenza1661	1970	C	T	het	het	catcccgccGtttcctcC	catcccgccGtttcctcT	gctcgccgatgaagagcT
IWGSC_CSS_1BL_scaff_3828996	Cadenza1661	1340	G	A	hom	hom	agccggatgttagtgttaacC	agccggatgttagtgttaacT	agcagcttgTcgcgttaaC
IWGSC_CSS_1DS_scaff_1884529	Cadenza1661	10575	G	A	hom	hom	aCagatacaAttgtcatgcaggC	aCagatacaAttgtcatgcaggT	acctgggTTgtccaatacttC
IWGSC_CSS_2AL_scaff_6318370	Cadenza1661	19142	C	T	het	—	cgtggcCgaatCtcGacG	cgtggcCgaatCtcGacA	ttcttgtgggagccgggC
IWGSC_CSS_2AS_scaff_5213460	Cadenza1661	1358	G	A	hom	hom	gtcacgaaCccgctcagG	gtcacgaaCccgctcagA	aggaaagagaggaaaagaGcG
IWGSC_CSS_2BS_scaff_5179331	Cadenza1661	5604	G	A	het	het	actctcgtcaagaactgatacaG	actctcgtcaagaactgatacaA	gcaGagaatgttcttgcaacT
IWGSC_CSS_2DS_scaff_5341235	Cadenza1661	4673	G	A	het	het	ggtgaggatctcggagctG	ggtgaggatctcggagctA	gcgcggtcgtacgagttG
IWGSC_CSS_3AL_scaff_4250995	Cadenza1661	7046	G	A	hom	hom	cCaagaaacgggtggtccaG	cCaagaaacgggtggtccaA	ctgcagctgtcccatcatcgT
IWGSC_CSS_3B_scaff_10404421	Cadenza1661	4303	G	A	het	het	ccttcgtcgaCaggacctG	ccttcgtcgaCaggacctA	GCcagtactCacAtgctctC
IWGSC_CSS_5DL_scaff_2390496	Cadenza1538	2125	C	T	hom	het	gcagttttatcctcagtagtcttgG	gcagttttatcctcagtagtcttgA	ttctgagaaTgtaatgtgcGatG
IWGSC_CSS_6AL_scaff_5753680	Cadenza1538	3920	C	T	hom	hom	tgctccaaatttgagcacaaTaaC	tgctccaaatttgagcacaaTaaT	aaatgcaaggggtaagttttttgT
IWGSC_CSS_6AS_scaff_4425792	Cadenza1538	4307	G	A	hom	het	agatgcttgtCggGccaG	agatgcttgtCggGccaA	gctgaagcaacgcgatcaaT
IWGSC_CSS_6BS_scaff_3003630	Cadenza1538	6933	C	T	het	het	ggcagtaatgtggtgctgagC	ggcagtaatgtggtgctgagT	tTgaCttctggtttggtggcA
IWGSC_CSS_6DL_scaff_3246988	Cadenza1538	9186	G	A	het	het	gctaaagaagagcttgagagaattC	gctaaagaagagcttgagagaattT	aatttctgaagagaggtgttgtatG
IWGSC_CSS_7AL_scaff_4480114	Cadenza1538	3446	C	T	het	—	gatatctcccacacggcgG	gatatctcccacacggcgA	tgagccactcttgcagtttT
IWGSC_CSS_7AS_scaff_4193541	Cadenza1538	8359	C	T	hom	het	agcaattctttggctatcaattagC	agcaattctttggctatcaattagT	tcatctGtcttaactctactgctG
IWGSC_CSS_7BL_scaff_6721572	Cadenza1538	9223	C	T	het	het	gctCagggaggaagacaagaaG	gctCagggaggaagacaagaaA	tgctatgaagaattccgacctC
IWGSC_CSS_7BS_scaff_3152545	Cadenza1538	3960	G	A	hom	—	tcagcaaaatcacctgcCgC	tcagcaaaatcacctgcCgT	gCtgccccatcatcgtttaT
IWGSC_CSS_7DS_scaff_3963838	Cadenza1538	2913	G	A	het	het	tCgttgcaagcCttTtgtgC	tCgttgcaagcCttTtgtgT	agaGttaTcaagcTactgtcacA
IWGSC_CSS_1AL_scaff_3903380	Cadenza1469	6193	G	A	hom	hom	ctcttcAgagatgaacgcgG	ctcttcAgagatgaacgcgA	tcGtGagatgGtggtttGTtA
IWGSC_CSS_1AS_scaff_3287728	Cadenza1469	3817	C	T	het*	hom	ccgaccaAttcactaaccgG	ccgaccaAttcactaaccgA	accctctttcccAgacatgaT
IWGSC_CSS_1BL_scaff_3815304	Cadenza1469	513	G	A	hom	hom	aacatttgcctTaCcaaaacGC	aacatttgcctTaCcaaaacGT	acacagcaagttataatgCAAgC
IWGSC_CSS_1DL_scaff_2266648	Cadenza1469	5926	C	T	het	het	caacatgagacacaacaccttC	caacatgagacacaacaccttT	gtcaacgcgtgaggattgtC
IWGSC_CSS_1DS_scaff_1906671	Cadenza1469	3697	C	T	hom	hom	tggTGtagacacttggcgaG	tggTGtagacacttggcgaA	catggcgaccaccAcctG
IWGSC_CSS_2AL_scaff_6337088	Cadenza1469	7334	G	A	het*	hom	acaatgccAagttgacaggttG	acaatgccAagttgacaggttA	gggagtgttggttCagaacaT

IWGSC contig	Line	Pos	WT	Mut	Predicted	M_4	Primer 1 (Cadenza)	Primer 2 (mutant)	Common Primer
IWGSC_CSS_2BL_scaff_7972799	Cadenza1469	8995	C	T	het	hom	gTgCtcctcGgcatccttC	gTgCtcctcGgcatccttT	gatccgGgcaaactacgTG
IWGSC_CSS_2DL_scaff_9832343	Cadenza1469	3262	G	A	het	het	TtgtctaAcagcacCGcagG	TtgtctaAcagcacCGcagA	agatctcggtcagcctttcT
IWGSC_CSS_2DS_scaff_5327939	Cadenza1469	3889	G	A	het	het	ttttTgccttatgtgactctagtaC	ttttTgccttatgtgactctagtaT	gaggccatcacagatagcG
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IWGSC_CSS_3B_scaff_10592217	Cadenza0580	2994	C	T	het	—	acagcagtatcaagcccctC	acagcagtatcaagcccctT	tgatactgttgTggCggagG
IWGSC_CSS_3DS_scaff_2596771	Cadenza0580	1037	G	A	het	het	tggttatgCAcaggataatCagG	tggttatgCAcaggataatCagA	tggcaaatgtgatgtcattaggT
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IWGSC_CSS_4DL_scaff_14359838	Cadenza0580	1408	G	A	hom	—	gCtcAttcagggatTGTcCtaTatG	gCtcAttcagggatTGTcCtaTatA	tgaCagaacagttggtcatacT
IWGSC_CSS_4DS_scaff_2276484	Cadenza0580	8034	G	A	hom	hom	gccgtggttgatggAgaG	gccgtggttgatggAgaA	cgtccagattactgatacttgcA
IWGSC_CSS_5AL_scaff_2756579	Cadenza0580	5278	G	A	het	het	tgaatggatttttcgtcccgttC	tgaatggatttttcgtcccgttT	ggAAtCCTATgCAgaAgAaaCTG
IWGSC_CSS_5BL_scaff_10787208	Cadenza0580	10627	G	A	het	—	gcctctcacatgcggagaC	gcctctcacatgcggagaT	acgatgtcAggtggGcgT
IWGSC_CSS_5BS_scaff_2282179	Cadenza0580	5267	G	A	het	—	tgatgggctacgacgtgC	tgatgggctacgacgtgT	tcggcgcccttgaaAtcC
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IWGSC_CSS_6AL_scaff_5757109	Cadenza0423	2788	G	A	hom	hom	caggaGcctggcaaataaaGG	caggaGcctggcaaataaaGA	ctttcGcagtctcttagtttcG
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IWGSC_CSS_6BL_scaff_4271391	Cadenza0423	4660	C	T	hom	hom	tacgtgcatgatgtggtagtcgtaC	tacgtgcatgatgtggtagtcgtaT	gtttgaagtgcatcagatgTaccA
IWGSC_CSS_6DS_scaff_1880206	Cadenza0423	9159	G	A	het	het	ctgCgaaggctccacaaG	ctgCgaaggctccacaaA	ggatgagaagtttgcattgctC
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IWGSC_CSS_7DS_scaff_407428	Cadenza0423	2051	G	A	het	het	gtcGCgccatcctgacaG	gtcGCgccatcctgacaA	actcatcAggtcagcccaA
IWGSC_CSS_3AL_scaff_442479	Cadenza0364	3198	C	T	het	het	gagtcaTtaagttggtaagattggC	gagtcaTtaagttggtaagattggT	GCaGaTaaCaacaggatcacG
IWGSC_CSS_3AL_scaff_4447942	Cadenza0364	11917	G	A	het	het	gtcataaagattgctcctgtgaaG	gtcataaagattgctcctgtgaaA	ctcGgatgtgggaggaagA
IWGSC_CSS_3AS_scaff_1557483	Cadenza0364	2547	C	T	het	het	aaagtcacatcatgcttaccataaG	aaagtcacatcatgcttaccataaA	cgaaatccaacgcctcatcA
IWGSC_CSS_3AS_scaff_2648747	Cadenza0364	2688	G	A	het	het	tggAagcAcaaggggccC	tggAagcAcaaggggccT	GccgccgatggagactcG
IWGSC_CSS_3AS_scaff_3304956	Cadenza0364	1017	G	A	het	het	gtcccttgcacacagctttG	gtcccttgcacacagctttA	cctgctggactacaacttcaaT
IWGSC_CSS_3AS_scaff_3321091	Cadenza0364	4585	C	T	het	het	caagaatgATgctgatgttggaG	caagaatgATgctgatgttggaA	acatgctgaatcgccgaatC
IWGSC_CSS_3AS_scaff_3371333	Cadenza0364	538	G	A	het	het	gggaaaCgAgAcgagcgG	gggaaaCgAgAcgagcgA	ccgtgccttcctcacccT
IWGSC_CSS_3AS_scaff_3371815	Cadenza0364	1061	C	T	het	het	atccccacggcacagagG	atccccacggcacagagA	aAttggcccttggtgattcC
IWGSC_CSS_3AS_scaff_3440912	Cadenza0364	4498	G	A	het	het	ccgtaaaactttctgtgcttgC	ccgtaaaactttctgtgcttgT	atActgacaaactacatgatgtgC
IWGSC_CSS_3B_scaff_10343586	Cadenza0364	2242	G	A	het	—	ggttcTgTcctctcttccactG	ggttcTgTcctctcttccactA	tgtgttgaacccgcaagcA
IWGSC_CSS_3AL_scaff_442479	Cadenza0364	3198	C	T	het	het	gagtcaTtaagttggtaagattggC	gagtcaTtaagttggtaagattggT	GCaGaTaaCaacaggatcacG
IWGSC_CSS_3AL_scaff_4447942	Cadenza0364	11917	G	A	het	het	gtcataaagattgctcctgtgaaG	gtcataaagattgctcctgtgaaA	ctcGgatgtgggaggaagA
IWGSC_CSS_3AS_scaff_1557483	Cadenza0364	2547	C	T	het	het	aaagtcacatcatgcttaccataaG	aaagtcacatcatgcttaccataaA	cgaaatccaacgcctcatcA
IWGSC_CSS_3AS_scaff_2648747	Cadenza0364	2688	G	A	het	het	tggAagcAcaaggggccC	tggAagcAcaaggggccT	GccgccgatggagactcG
IWGSC_CSS_3AS_scaff_3304956	Cadenza0364	1017	G	A	het	het	gtcccttgcacacagctttG	gtcccttgcacacagctttA	cctgctggactacaacttcaaT
IWGSC_CSS_3AS_scaff_3321091	Cadenza0364	4585	C	T	het	het	caagaatgATgctgatgttggaG	caagaatgATgctgatgttggaA	acatgctgaatcgccgaatC

IWGSC contig	Line	Pos	WT	Mut	Predicted	M_4	Primer 1 (Cadenza)	Primer 2 (mutant)	Common Primer
IWGSC_CSS_3AS_scaff_3371333	Cadenza0364	538	G	A	het	het	gggaaaCgAgAcgagcgG	gggaaaCgAgAcgagcgA	ccgtgccttcctcacccT
IWGSC_CSS_3AS_scaff_3371815	Cadenza0364	1061	C	T	het	het	atccccacggcacagagG	atccccacggcacagagA	aAttggccctggtgattcC
IWGSC_CSS_3AS_scaff_3440912	Cadenza0364	4498	G	A	het	het	ccgtaaaactttctgtgcttgC	ccgtaaaactttctgtgcttgT	atActgacaaactacatgatgtgC
IWGSC_CSS_3B_scaff_10343586	Cadenza0364	2242	G	A	het	—	ggttcTgTcctctcttccactG	ggttcTgTcctctcttccactA	tgtgttgaacccgcaagcA
IWGSC_CSS_5DL_scaff_242342	Cadenza0281	2433	C	T	hom	hom	catggCgacggtGtcctG	catggCgacggtGtcctA	aAccctcatTTtggCTACTtCT
IWGSC_CSS_5DL_scaff_4538822	Cadenza0281	1208	G	A	hom	—	acgtcagaacaaccgtttgaC	acgtcagaacaaccgtttgaT	ttaaattggttggcgccacC
IWGSC_CSS_6AL_scaff_5813297	Cadenza0281	4532	C	T	hom	—	gggagagggacgtctcgG	gggagagggacgtctcgA	ttcttctgccaacgattccG
IWGSC_CSS_6AS_scaff_4378990	Cadenza0281	6748	C	T	hom	hom	cccaggttctgcttcttttcC	cccaggttctgcttcttttcT	caagtatcaagaaaatgaagggTgT
IWGSC_CSS_6BL_scaff_4360781	Cadenza0281	5426	C	T	het	het	aCtactcaaatggcttGgtgtaG	aCtactcaaatggcttGgtgtaA	tcagtccaacatgTcaagagatT
IWGSC_CSS_7AL_scaff_4488310	Cadenza0281	3808	G	A	hom	hom	gttctcttgtagtagcagccG	gttctcttgtagtagcagccA	ggcgctttcttcggcctA
IWGSC_CSS_7BL_scaff_6696509	Cadenza0281	9232	G	A	het	het	gctctaggGgtggcaaAagG	gctctaggGgtggcaaAagA	ggcttGaGgtcGcagtgT
IWGSC_CSS_7BS_scaff_3143575	Cadenza0281	1866	C	T	het	het	agatgttgagagggcgcttC	agatgttgagagggcgcttT	gcttggAtggtggcaagtT
IWGSC_CSS_7DL_scaff_3346250	Cadenza0281	1663	G	A	het	het	acgtgcagcaacatcctaaC	acgtgcagcaacatcctaaT	TttcccaccaggcccaagA
IWGSC_CSS_7DS_scaff_3933917	Cadenza0281	1243	C	T	het	het	tgCtgagcCttTcaccttgC	tgCtgagcCttTcaccttgT	agaggtttggttccatcGG
IWGSC_CSS_3B_scaff_10626860	Cadenza0148	7847	G	A	het	het	gcagctctgggaaggagG	gcagctctgggaaggagA	gttaatgtacCTcctagcctcG
IWGSC_CSS_3DL_scaff_6915683	Cadenza0148	6904	C	T	het	het	cgtcaaCctgtgggcaattG	cgtcaaCctgtgggcaattA	tcatgctcataatgTcatagggT
IWGSC_CSS_4AS_scaff_5929057	Cadenza0148	4238	G	A	hom	hom	gcgcaacgtagCacctacC	gcgcaacgtagCacctacT	ttatctggtgaagtgacaggttCA
IWGSC_CSS_4AS_scaff_5950625	Cadenza0148	10590	C	T	het	het	agaTattCaaaTcggtggAttggC	agaTattCaaaTcggtggAttggT	cctgCtcccctcacgtcC
IWGSC_CSS_4AS_scaff_5967119	Cadenza0148	11626	C	T	hom	hom	cgtGgacaccccgagctG	cgtGgacaccccgagctA	gacgacgcactgcacgaC
IWGSC_CSS_4DL_scaff_14455742	Cadenza0148	1946	C	T	hom	hom	gCctgagggagatcgcgC	gCctgagggagatcgcgT	aaccgGtAaCTGtGgGcA
IWGSC_CSS_4DS_scaff_2318993	Cadenza0148	4000	C	T	hom	hom	tccagtttgacacagattgaatggG	tccagtttgacacagattgaatggA	tgagaTtctgtttcctttcacAttG
IWGSC_CSS_5AL_scaff_2750707	Cadenza0148	4603	G	A	het	het	ccttggtgctagccatttcaagTaG	ccttggtgctagccatttcaagTaA	ccaggaTgcAgtgcaatatttcaaG
IWGSC_CSS_5BL_scaff_10794137	Cadenza0148	9235	C	T	hom	hom	gaagctgcttctgcgttG	gaagctgcttctgcgttA	agtatcccttccatataagcagtG
IWGSC_CSS_5BS_scaff_1646558	Cadenza0148	2916	C	T	het	het	gccGtacactcacctAtcctttG	gccGtacactcacctAtcctttA	gcaaTgtccacttAtcatcccT
IWGSC_CSS_1AL_scaff_3883106	Cadenza0110	27536	C	T	het	het	accttccatcactggctgG	accttccatcactggctgA	gtgaagaacaacaggttgaagC
IWGSC_CSS_1BL_scaff_3812829	Cadenza0110	10770	G	A	het*	hom	cccccactccattccagG	cccccactccattccagA	gGatgttgttctgtgctggaA
IWGSC_CSS_1DL_scaff_2266648	Cadenza0110	6156	G	A	het	het	actgcgtggttatgggacC	actgcgtggttatgggacT	ccccatcactgaacacaacA
IWGSC_CSS_1DS_scaff_1889435	Cadenza0110	8826	C	T	hom	hom	aaccatgaattactcggacagG	aaccatgaattactcggacagA	gccctgaagaattgtatcaaaacaG
IWGSC_CSS_2AS_scaff_5268634	Cadenza0110	4636	G	A	het	het	gatccatgtgattggcatgtttG	gatccatgtgattggcatgtttA	TgctgtTggatatgcagttacT
IWGSC_CSS_2BL_scaff_7965110	Cadenza0110	15801	C	T	hom	hom	cattgaagcAtacacAattgcAtaC	cattgaagcAtacacAattgcAtaT	gccagagtatccagataaggTttA
IWGSC_CSS_2DL_scaff_9852812	Cadenza0110	13788	G	A	hom	hom	atttttgtatggtctcaatcttcgC	atttttgtatggtctcaatcttcgT	gaacgtTcattcttgtacttgcT
IWGSC_CSS_2DS_scaff_5371379	Cadenza0110	2166	C	T	hom	hom	agacacaaaactagtGatgcgC	agacacaaaactagtGatgcgT	gctgctgagaatgttTtgtatttG
IWGSC_CSS_3AL_scaff_4384278	Cadenza0110	1276	C	T	het	het	agcTgaactgccccTgtaG	agcTgaactgccccTgtaA	agggacctCgGtggatgaA
IWGSC_CSS_3AS_scaff_3340122	Cadenza0110	1467	C	T	hom	hom	attcctAgtgttgtcggaacatG	attcctAgtgttgtcggaacatA	gagaagactagaaagttttcAgcaT
IWGSC_CSS_5DL_scaff_4554222	Cadenza2103	6528	C	T	het*	hom	gctgccctacaaagaaacaaaattG	gctgccctacaaagaaacaaaattA	aTcccaactatCGaTtttgtcataC
IWGSC_CSS_6AL_scaff_5833640	Cadenza2103	7346	C	T	hom	hom	aagaaaagccacaatggtttctC	aagaaaagccacaatggtttctT	aCTctgTcagtgtttcccagC
IWGSC_CSS_6AS_scaff_4429974	Cadenza2103	3867	G	A	hom	hom	GagatgaAtttattgagcatgtggC	GagatgaAtttattgagcatgtggT	ggttccggctgcataagT
IWGSC_CSS_6DL_scaff_3307626	Cadenza2103	4970	C	T	hom	hom	tgcagatgttgtcctgtgtaG	tgcagatgttgtcctgtgtaA	ctaggaaggtgattttgtactGtC
IWGSC_CSS_6DS_scaff_2059604	Cadenza2103	5224	G	A	het	—	gctcaatgcatgcTgagtgG	gctcaatgcatgcTgagtgA	tgtcaagtattattttcctgctctG
IWGSC_CSS_7AL_scaff_4552322	Cadenza2103	1412	C	T	het	het	gcaaaggcTgatactccaacaG	gcaaaggcTgatactccaacaA	ggcAAGccAgtataaaagtaaGC

IWGSC contig	Line	Pos	WT	Mut	Predicted	M_4	Primer 1 (Cadenza)	Primer 2 (mutant)	Common Primer
IWGSC_CSS_7BS_scaff_3147455	Cadenza2103	4607	G	A	het	—	gcaccttaggatgtgagTtatgC	gcaccttaggatgtgagTtatgT	gcatgtagggtttatttgactgttA
IWGSC_CSS_7DL_scaff_3382467	Cadenza2103	3473	C	T	hom	—	GGTtctgCaGTTCATAActcatC	GGTtctgCaGTTCATAActcatT	attgaatcaactgatacGaaGactC
IWGSC_CSS_3B_scaff_10457010	Cadenza0277	10599	G	A	het	het	aaccttggccgcagaacaC	aaccttggccgcagaacaT	actggctgcacgagaggG
IWGSC_CSS_3B_scaff_10593852	Cadenza0277	10124	C	T	het	het	tgacaggggacgctatacaG	tgacaggggacgctatacaA	gtctaaCTtACattAcccatcagC
IWGSC_CSS_3DS_scaff_2583390	Cadenza0277	663	G	A	hom	hom	actgcactcatacaatActtCtgC	actgcactcatacaatActtCtgT	tcCacctggacagcaagtG
IWGSC_CSS_4AL_scaff_7093953	Cadenza0277	10004	C	T	hom	hom	ccttgtattcaatggaTtgTtttgG	ccttgtattcaatggaTtgTtttgA	ttccccaaaTaaaaaggaagagC
IWGSC_CSS_4AL_scaff_7176064	Cadenza0277	6220	C	T	het	het	gtgccgtaTtcCgcctgG	gtgccgtaTtcCgcctgA	atgttcgaggggatgggG
IWGSC_CSS_4DL_scaff_14122349	Cadenza0277	1010	C	T	hom	hom	gtcgctgctgCttgtgaG	gtcgctgctgCttgtgaA	ggaacaggcccaaggagG
IWGSC_CSS_5AL_scaff_2736916	Cadenza0277	4296	G	A	het	het	aagaactATgAaaGtaacacacgaC	aagaactATgAaaGtaacacacgaT	ttcGcTttTaagGcAttCtcG
IWGSC_CSS_5BL_scaff_10883744	Cadenza0277	2080	C	T	hom	hom	gcctctttCtgttTagcctcaG	gcctctttCtgttTagcctcaA	cgacaaggttcgtgatTgcA
IWGSC_CSS_1AL_scaff_3932013	Cadenza0548	11765	C	T	hom	hom	accgccaaCccaagacaG	accgccaaCccaagacaA	cccattaGccgTgcAacG
IWGSC_CSS_1BS_scaff_3417505	Cadenza0548	373	C	T	het	het	gtggtgaggaGGgtgGaG	gtggtgaggaGGgtgGaA	tggtcgGccagttgttgA
IWGSC_CSS_2AS_scaff_5305619	Cadenza0548	2786	C	T	hom	hom	atacagatgccctAAgtggTtC	atacagatgccctAAgtggTtT	ggaagacaAtGctccaggtaC
IWGSC_CSS_2AS_scaff_5306489	Cadenza0548	46953	T	G	het	wt	aggttccatgtccatagaagGT	aggttccatgtccatagaagGG	aggctaTAgactcctgtACAgT
IWGSC_CSS_2BL_scaff_7984123	Cadenza0548	11660	G	A	het	het	cattgtggcatagtaatcagtacaG	cattgtggcatagtaatcagtacaA	aatacattgaggaatcaaagccC
IWGSC_CSS_2DL_scaff_9907477	Cadenza0548	1363	C	T	hom	hom	tgcctccctttgccagaaC	tgcctccctttgccagaaT	ggcaaacctgatgtggcatC
IWGSC_CSS_2DS_scaff_5330886	Cadenza0548	5449	G	A	hom	hom	gcatgtccatttatactgaaCgtG	gcatgtccatttatactgaaCgtA	catgctgcttcttctggacC
IWGSC_CSS_3AL_scaff_4449951	Cadenza0548	633	C	T	het	het	tccaaacctaacagtctaacactaG	tccaaacctaacagtctaacactaA	gtctgcagTGCaatgtgC
IWGSC_CSS_3B_scaff_10479889	Cadenza0097	3339	C	T	hom	—	ttgTttctGgagaagatgcCG	ttgTttctGgagaagatgcCA	ggtgctcattcaAcGgcA
IWGSC_CSS_3B_scaff_10562262	Cadenza0097	7819	C	T	het	het	agaggggtgctatccatAttgG	agaggggtgctatccatAttgA	agcgatgccaaggcttcC
IWGSC_CSS_4AL_scaff_7040796	Cadenza0097	10772	G	A	hom	hom	acacaacattgccaccagaG	acacaacattgccaccagaA	CAatCgattgcttgctTctcC
IWGSC_CSS_4AL_scaff_7063488	Cadenza0097	6360	C	T	het	het	gcctctcacCttAatttgaagctgC	gcctctcacCttAatttgaagctgT	aggcagtggagtatgtgaagttT
IWGSC_CSS_4AL_scaff_7091701	Cadenza0097	5050	G	A	het	het	catgagcatctgggaggaaaatG	catgagcatctgggaggaaaatA	agcaagggaAtaatgaacggaaA
IWGSC_CSS_4DS_scaff_1845841	Cadenza0097	7110	G	A	hom	hom	aatgTAgctccccatacCgG	aatgTAgctccccatacCgA	actgaaacTgcaatcgtTtatggA
IWGSC_CSS_5AL_scaff_2767581	Cadenza0097	3737	G	A	het	het	gagaggtcctcactAtcggC	gagaggtcctcactAtcggT	cgTcatcacaaatattgctggG
IWGSC_CSS_5BL_scaff_10784643	Cadenza0097	1568	C	T	hom	hom	agaaaTAcatggatggatggaCG	agaaaTAcatggatggatggaCA	catctcCCttccaCgGaaaG
IWGSC_CSS_1AL_scaff_3952258	Cadenza2092	8107	C	T	het	—	tgagtagagaaattgacagtgtgG	tgagtagagaaattgacagtgtgA	tgccaccattgacatgagaG
IWGSC_CSS_1BL_scaff_3858008	Cadenza2092	10278	G	A	hom	hom	tttgagcaggcaggatcgC	tttgagcaggcaggatcgT	actcacggcctatatcActattC
IWGSC_CSS_1DL_scaff_2265172	Cadenza2092	9094	C	T	hom	hom	tgcaTGTcatttgttcttatcagC	tgcaTGTcatttgttcttatcagT	agtgtccaacttccGttcatC
IWGSC_CSS_2AL_scaff_6435867	Cadenza2092	16201	G	A	hom	hom	tttctgTaccttaacgtcaattgaC	tttctgTaccttaacgtcaattgaT	gtgaggatgatgaggtaagacC
IWGSC_CSS_2AL_scaff_6439430	Cadenza2092	25101	C	T	het	—	caagaaagggCagCtCagC	caagaaagggCagCtCagT	tcGttAcTctttcActggtgaA
IWGSC_CSS_2DL_scaff_9760848	Cadenza2092	4733	C	T	het	het	gcaccatgggtctcaggtaC	gcaccatgggtctcaggtaT	tcagtcagtttGCTCtgTCTG
IWGSC_CSS_3AL_scaff_4407012	Cadenza2092	2785	C	T	hom	hom	acatatAgtgttctcatccaccatC	acatatAgtgttctcatccaccatT	acctctctcatgttaataggtttgT
IWGSC_CSS_3AS_scaff_3441108	Cadenza2092	541	G	A	het	het	GtgatgaccttgagacGgaG	GtgatgaccttgagacGgaA	aggcaTgacaaCgcgcaA
IWGSC_CSS_3B_scaff_10449827	Cadenza1551	4779	G	A	hom	hom	ggcaaggtcaagaaacGgtC	ggcaaggtcaagaaacGgtT	aCagaGtgggttagaggcaG
IWGSC_CSS_3B_scaff_10550638	Cadenza1551	3250	C	T	het	het	ctccttcacttgttgcggC	ctccttcacttgttgcggT	gcaacAtTttgatactgcaaagG
IWGSC_CSS_3DL_scaff_6945816	Cadenza1551	589	C	T	hom	hom	agcatctcacctgcaaCaataC	agcatctcacctgcaaCaataT	TgtgcccTctgaAtattttcaTG
IWGSC_CSS_3DL_scaff_6954177	Cadenza1551	3508	C	T	het	het	tgtagcatcacattaactttcctG	tgtagcatcacattaactttcctA	gcttggtataaaccCttacgacA
IWGSC_CSS_4AS_scaff_5938272	Cadenza1551	19080	G	A	hom	hom	agAcCccgAtcgccatgG	agAcCccgAtcgccatgA	GggAgatAcaggtaaaActcTtcG
IWGSC_CSS_4AS_scaff_5977594	Cadenza1551	11092	C	T	het	het	gccttgattcggaacaacaaaC	gccttgattcggaacaacaaaT	gcgtctctcagtcctgcA

IWGSC contig	Line	Pos	WT	Mut	Predicted	M_4	Primer 1 (Cadenza)	Primer 2 (mutant)	Common Primer
IWGSC_CSS_5AL_scaff_2671035	Cadenza1551	5859	C	T	het	het	cggtgatattTttagacttcgacgC	cggtgatattTttagacttcgacgT	ggcagttcagcGacccatT
IWGSC_CSS_5BL_scaff_10889480	Cadenza1551	2530	G	A	hom	hom	gagcttaactcgcagatggaG	gagcttaactcgcagatggaA	tccatgCAacGccttggT
IWGSC_CSS_3B_scaff_10528396	Cadenza2088	8059	G	A	hom	—	cttttccgtccgtaagcaataG	cttttccgtccgtaagcaataA	gtgcactgttcaggcctgA
IWGSC_CSS_3B_scaff_10637573	Cadenza2088	16815	G	A	het	het	agcaagcttaccGgtctgC	agcaagcttaccGgtctgT	cgagcAactacgagcagctT
IWGSC_CSS_4AL_scaff_7086469	Cadenza2088	6697	G	A	het	het	gccgtctacttcaacgcG	gccgtctacttcaacgcA	ccaGaggcttgtTGcattttT
IWGSC_CSS_4AL_scaff_7126302	Cadenza2088	3627	G	A	hom	hom	gttcaaaaacaagtggctAatttgC	gttcaaaaacaagtggctAatttgT	cacaaggatatgaagcTcttctagA
IWGSC_CSS_4BL_scaff_7041808	Cadenza2088	10234	G	A	hom	hom	tcaatggatgagggtgcttC	tcaatggatgagggtgcttT	ccatagcagcatcagccacA
IWGSC_CSS_5AL_scaff_2794167	Cadenza2088	13162	G	A	het	—	agtattcaggacaagcatCttCaG	agtattcaggacaagcatCttCaA	caatgaaacctctcgaagaaGaG
IWGSC_CSS_5BL_scaff_10889232	Cadenza2088	3885	G	A	het	het	cTcaaccacaatgggcaAatC	cTcaaccacaatgggcaAatT	tccttcatcaatcatcaattgttgG
IWGSC_CSS_5BS_scaff_2267405	Cadenza2088	11113	C	T	hom	hom	ctttgatgatcctaggcctctTG	ctttgatgatcctaggcctctTA	tgatttggtCtggttAgagtttGA
IWGSC_CSS_3B_scaff_10475354	Cadenza1409	2203	G	A	hom	hom	agCgaacaagagGtcaaacG	agCgaacaagagGtcaaacA	ctgaaacacaCtagaCAattAccG
IWGSC_CSS_3B_scaff_10674115	Cadenza1409	4555	C	T	het	het	gcttcagtgcatgccttcaG	gcttcagtgcatgccttcaA	cttcacacccGagataatGtattG
IWGSC_CSS_4AL_scaff_7153568	Cadenza1409	13073	C	T	hom	hom	tccgaccgAtcaaccttgG	tccgaccgAtcaaccttgA	gaccggaactcctcggcC
IWGSC_CSS_4DL_scaff_14314966	Cadenza1409	2010	G	A	het	hom	gtaggtcccctcctCAggG	gtaggtcccctcctCAggA	cggcgTcacaAgttgCcT
IWGSC_CSS_4DS_scaff_2324074	Cadenza1409	7606	G	A	het	het	tGcatgaaaatgtgtGcaGaG	tGcatgaaaatgtgtGcaGaA	gggtaAgttcAaaactGaagtgaaG
IWGSC_CSS_5AS_scaff_1517889	Cadenza1409	3561	G	A	het	het	tctcgacatcttcccgtgtaC	tctcgacatcttcccgtgtaT	gtgcctggaacattgcttatttA
IWGSC_CSS_5AS_scaff_1523866	Cadenza1409	8054	G	A	hom	—	ggtgatctaccgccaGgaC	ggtgatctaccgccaGgaT	tcctgcagCcTctcctcA
IWGSC_CSS_5BL_scaff_10917655	Cadenza1409	19073	G	A	hom	hom	caaatgacatgcaaaagaagttgC	caaatgacatgcaaaagaagttgT	cgcttcatcactacaAaatatgtcT
IWGSC_CSS_1AL_scaff_3886649	Cadenza1599	5204	C	T	het	het	tgatgccaaccacaatGcC	tgatgccaaccacaatGcT	ggactgactgctgaccatatttaG
IWGSC_CSS_1BL_scaff_3810267	Cadenza1599	6634	C	T	hom	hom	ccCaggaaatgagcacctC	ccCaggaaatgagcacctT	cgcaggcgaagatgtgaTtG
IWGSC_CSS_1DL_scaff_2291677	Cadenza1599	12856	C	T	hom	hom	GgtagacaagtcgccgaG	GgtagacaagtcgccgaA	cctcctccttcaacGCcG
IWGSC_CSS_2AL_scaff_6354492	Cadenza1599	7566	G	A	het	het	gGagaatgcaCAgtAacTtctgG	gGagaatgcaCAgtAacTtctgA	ttccgaagaaccacaTccTG
IWGSC_CSS_2AS_scaff_5282937	Cadenza1599	9736	G	A	het	het	gctgtagattttatagctgctatgC	gctgtagattttatagctgctatgT	cacCagaattgttCactgatttTC
IWGSC_CSS_2BL_scaff_7952427	Cadenza1599	19249	G	A	hom	hom	cgTccctCcctagcacgaC	cgTccctCcctagcacgaT	aTcactccattagcgcgAG
IWGSC_CSS_2DL_scaff_9897981	Cadenza1599	5627	C	T	het	het	cttggtgctTgattgcttactC	cttggtgctTgattgcttactT	gTttgctCtctctgatctTtgtG
IWGSC_CSS_3AL_scaff_4446105	Cadenza1599	1765	G	A	hom	—	aaatgctttcctaCcgctagtG	aaatgctttcctaCcgctagtA	ttctAgaggcaatagctTatatgcT

Table A.2: Validation of mutations on M_4 on Kronos

IWGSC contig	Line	Pos	WT	Mut	Predicted	M_4	Primer 1 (Kronos)	Primer 2 (mutant)	Common Primer
IWGSC_CSS_1AS_scaff_3284790	Kronos3085	7449	G	A	Het	Het	ccacaccttgagcctcgC	ccacaccttgagcctcgT	gtgattttgccaggggagA
IWGSC_CSS_1BL_scaff_3897513	Kronos3085	1515	C	T	Het	Het	gcttccactGggtcctgC	gcttccactGggtcctgT	acAaggactgcttcagaGaC
IWGSC_CSS_2AL_scaff_6434745	Kronos3085	3424	C	T	Het	Het	cctcGgttttgcaaatttctatgC	cctcGgttttgcaaatttctatgT	gGCaaTggcataacaacagatA
IWGSC_CSS_3AS_scaff_3408995	Kronos3085	732	C	T	Het	Het	aggccatttcgaattccgC	aggccatttcgaattccgT	ggTgttaTccagAacctgagTG
IWGSC_CSS_3B_scaff_10708748	Kronos3085	2675	G	A	Het	Het	gttgcatgcttcacccagG	gttgcatgcttcacccagA	gtaacaatctgagttcgtagcaC
IWGSC_CSS_4AL_scaff_7132733	Kronos3085	1799	C	T	Hom	Hom	cacccgtgagtgaccctC	cacccgtgagtgaccctT	aCcGcctaGaaagaaagcttC
IWGSC_CSS_5AS_scaff_1534693	Kronos3085	4605	C	T	Het	Het	cagcttcctggccctcAtC	cagcttcctggccctcAtT	gtaCctcacgAgtcaTgagAG
IWGSC_CSS_6AS_scaff_4361911	Kronos3085	8857	G	A	Het	Het	tcacgaaagacgacttcaacctcC	tcacgaaagacgacttcaacctcT	catgaggtgctgcatctccatcA
IWGSC_CSS_6BS_scaff_3008326	Kronos3085	1528	G	A	Het	Het	ccatgttgtactggtggtgC	ccatgttgtactggtggtgT	ggaagcatggCaagtgcA
IWGSC_CSS_7AS_scaff_4214385	Kronos3085	27835	C	T	Hom	Hom	cgtaccttcgttgggaaagG	cgtaccttcgttgggaaagA	ctcttggtcagctgtataagacT
IWGSC_CSS_1AL_scaff_3929964	Kronos3191	1336	C	T	Het	Het	tttcggccatacctgacatC	tttcggccatacctgacatT	attgcctccagttcttgcaG
IWGSC_CSS_1BL_scaff_3899789	Kronos3191	7925	C	T	Het	Het	actctcacTggcagcagC	actctcacTggcagcagT	caacgtggtgcccatcGtA
IWGSC_CSS_2AL_scaff_6426728	Kronos3191	1481	G	A	Hom	Hom	gaaActgccgcagctCgC	gaaActgccgcagctCgT	ccaGcaGctcgtgagaaA
IWGSC_CSS_2BL_scaff_7960273	Kronos3191	690	C	T	Hom	Hom	gccattcatccttaggcgC	gccattcatccttaggcgT	acatgcaattgctgatgactG
IWGSC_CSS_3AS_scaff_3286603	Kronos3191	2975	G	A	Het*	Hom	ccgtgtggtttgttgtggG	ccgtgtggtttgttgtggA	gaaaggaacgtgTcaTgcaG
IWGSC_CSS_5AL_scaff_2694249	Kronos3191	2399	C	T	Het	Het	gccttccagatagagccGC	gccttccagatagagccGT	cgccacatcgacattcctG
IWGSC_CSS_5BL_scaff_10923577	Kronos3191	3713	C	T	Het	Het	gtggattgcctgagcttgC	gtggattgcctgagcttgT	tggtggccttcttgggaC
IWGSC_CSS_6AL_scaff_5823017	Kronos3191	13225	C	T	Hom	Hom	ccctttcgagcctctggaG	ccctttcgagcctctggaA	ttcgagaaggcccatcgA
IWGSC_CSS_6BS_scaff_2955394	Kronos3191	1622	C	T	Het*	Hom	gtggagatgaaggtctagcaaG	gtggagatgaaggtctagcaaA	gatactcgTgcaatgggtgT
IWGSC_CSS_7BL_scaff_6739382	Kronos3191	12261	G	A	Hom	Hom	gagacaagctttgaattgctcC	gagacaagctttgaattgctcT	CgagtgacctTcatttcccG
IWGSC_CSS_1AS_scaff_3276389	Kronos3288	9720	C	T	Hom	Hom	aCcaGcaggaccAatgtctC	aCcaGcaggaccAatgtctT	atgatgcaacctcagccaT
IWGSC_CSS_2AL_scaff_6367515	Kronos3288	6976	G	A	Het	Het	caggtcgagTgtctccgG	caggtcgagTgtctccgA	ggggtgatCtggaagggC
IWGSC_CSS_2AL_scaff_6422019	Kronos3288	4523	G	A	Het	Het	cgctaggtccctgcatagG	cgctaggtccctgcatagA	acgcAcgctaagccgtaC
IWGSC_CSS_3AL_scaff_4284850	Kronos3288	7901	C	T	Hom	Hom	tggctttggacaacatcgG	tggctttggacaacatcgA	tgtcAgcatcgacagccaG
IWGSC_CSS_4AS_scaff_5962359	Kronos3288	13049	G	A	Het	Hom	ccatcaagaagtacgagttcgaC	ccatcaagaagtacgagttcgaT	accatgcccagcttgtcA
IWGSC_CSS_6AL_scaff_5778773	Kronos3288	6853	G	A	Het	Het	gagtgaccttcccgtctttC	gagtgaccttcccgtctttT	ggagaacagctactcggcT
IWGSC_CSS_6AS_scaff_4392100	Kronos3288	3434	C	T	Het	Het	atggaagcacaggtgaccG	atggaagcacaggtgaccA	ggAagcgaaagtgaacaaacA
IWGSC_CSS_7BL_scaff_6744240	Kronos3288	9772	G	A	Het	Het	agctgttcttctcctacttcaaG	agctgttcttctcctacttcaaA	caggtcgttcttgagctcC
IWGSC_CSS_1AL_scaff_3887185	Kronos3413	9708	C	T	Hom	Hom	gcacgcctttatcgaggtaaaG	gcacgcctttatcgaggtaaaA	AgaaacagcagagcgcaA
IWGSC_CSS_2BS_scaff_3381362	Kronos3413	5160	C	T	Het*	Hom	caacttctgggctgtagtgtG	caacttctgggctgtagtgtA	tgAgaattctgacGcaaaagaC
IWGSC_CSS_3AS_scaff_3296605	Kronos3413	6154	G	A	Het	Het	ctggtcacgggctctagC	ctggtcacgggctctagT	cagcactgagagacatggaC
IWGSC_CSS_3B_scaff_10693516	Kronos3413	12632	C	T	Het	Het	ctaggcttggacaaacaggC	ctaggcttggacaaacaggT	agcttgcatctatgggcatT
IWGSC_CSS_5AS_scaff_1547699	Kronos3413	2686	G	A	Het	Het	gCtacaaccttcaccaatcgC	gCtacaaccttcaccaatcgT	gacggctttgaagtgtcatC
IWGSC_CSS_5BL_scaff_10856077	Kronos3413	5853	G	A	Het	Het	agagcttcaccccatgctC	agagcttcaccccatgctT	acgCacatttAatagctgaagC
IWGSC_CSS_6AL_scaff_5750718	Kronos3413	11046	G	A	Hom	Hom	cacgcTtcccgacttcttataG	cacgcTtcccgacttcttataA	AgacgatgtgatcaggattcaG
IWGSC_CSS_7AL_scaff_4433177	Kronos3413	3511	C	T	Het	Het	GaTgctccGtcaggctgG	GaTgctccGtcaggctgA	cactactggacaagctcttgG

IWGSC contig	Line	Pos	WT	Mut	Predicted	M_4	Primer 1 (Kronos)	Primer 2 (mutant)	Common Primer
IWGSC_CSS_7BL_scaff_6742567	Kronos3413	667	C	T	Het	Het	gttgcttgcgtggcagaC	gttgcttgcgtggcagaT	cattttgcaccgtgtgtcTG
IWGSC_CSS_1AL_scaff_3976389	Kronos3935	10941	C	T	Hom	Hom	ggtgaggagatcggCgatG	ggtgaggagatcggCgatA	cagtcatctacatgagaggtcaG
IWGSC_CSS_1BL_scaff_3873362	Kronos3935	1392	G	A	Het	Het	cagatctgaagcctaGcacatG	cagatctgaagcctaGcacatA	actaccagaatcagcacaaaaAC
IWGSC_CSS_2BL_scaff_7882382	Kronos3935	2721	C	T	Het	Het	gcaagctaagatgtaccgtagC	gcaagctaagatgtaccgtagT	gccacagtaggagaaagactT
IWGSC_CSS_3AL_scaff_4242376	Kronos3935	2410	C	T	Het	Het	agaacccaaaaccgTacttaG	agaacccaaaaccgTacttaA	gtagGgtCcatcCtaaagcttG
IWGSC_CSS_3B_scaff_10485067	Kronos3935	3349	C	T	Hom	Hom	gcttgagcaactactccaactG	gcttgagcaactactccaactA	gcaatttcctttaTccgcagT
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IWGSC_CSS_6BS_scaff_3045205	Kronos3935	2293	G	A	Het	Het	aaggaccaagcccaaactctcG	aaggaccaagcccaaactctcA	agtgatcaagcccaatgtcgcA
IWGSC_CSS_7AL_scaff_4555249	Kronos3935	4487	C	T	Het	Het	cAgtgctcgagatggcgC	cAgtgctcgagatggcgT	cCttgcaaccctcctgatT
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IWGSC_CSS_7BL_scaff_6716931	Kronos4240	2613	G	A	Het	Het	gGtgGgtattTgcttggtgaG	gGtgGgtattTgcttggtgaA	tgGtggactcgacaGtGtA
IWGSC_CSS_2BL_scaff_8029221	Kronos4346	2860	G	A	Het	Het	tgcttccgctcttgctcC	tgcttccgctcttgctcT	atTtgcatTCgAtcgggcC
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IWGSC_CSS_3B_scaff_10775915	Kronos4485	4701	C	T	Het	Het	ccaagggctgcagagagG	ccaagggctgcagagagA	agacctcacgatGtcctcC
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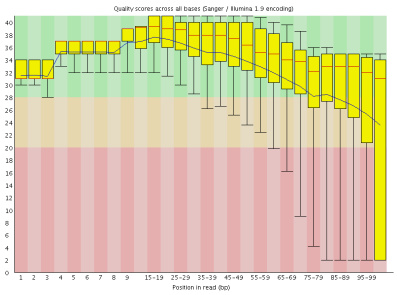
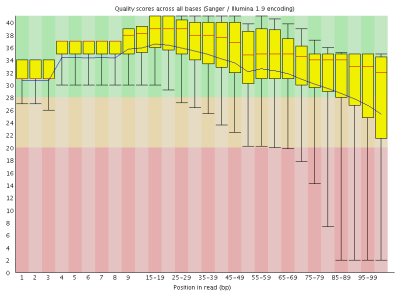
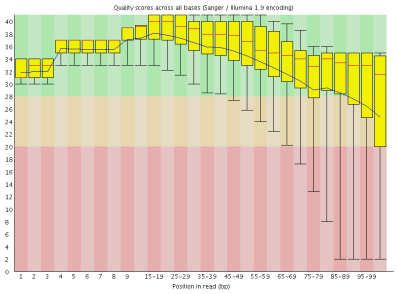
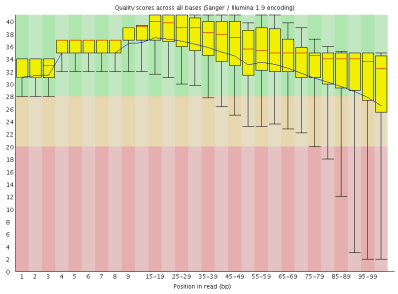
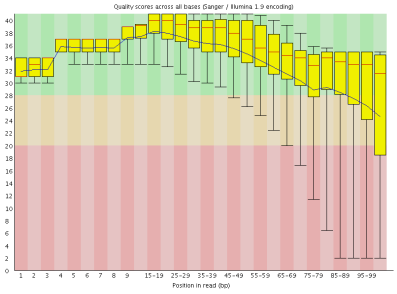
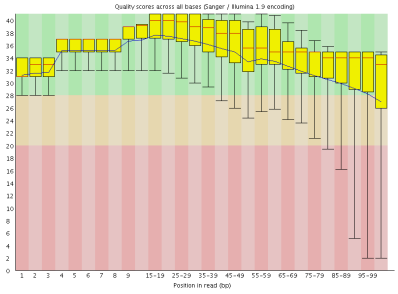
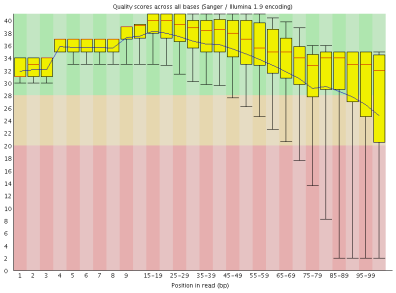
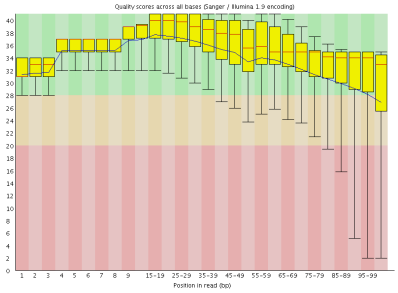
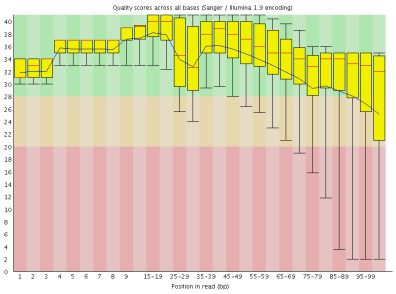
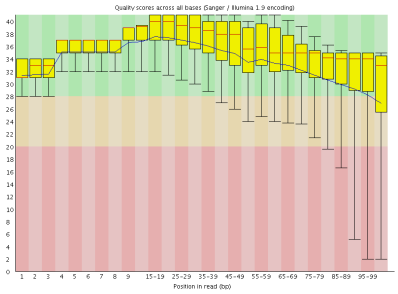
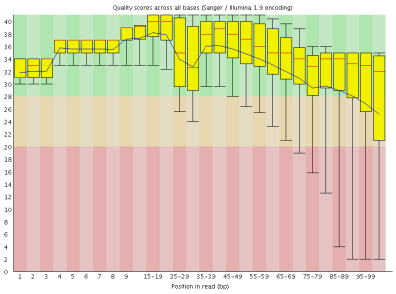
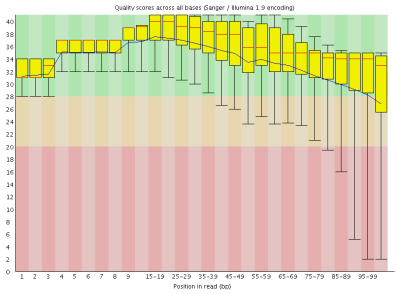
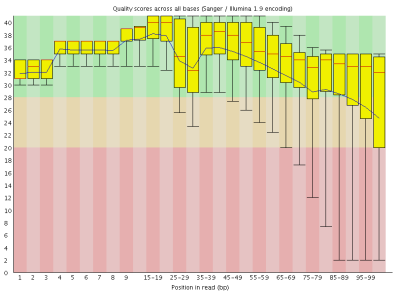
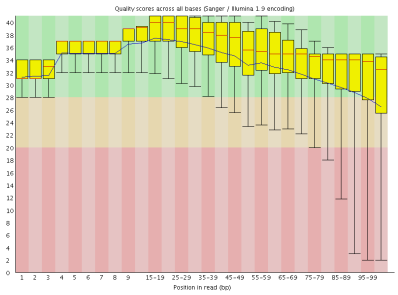
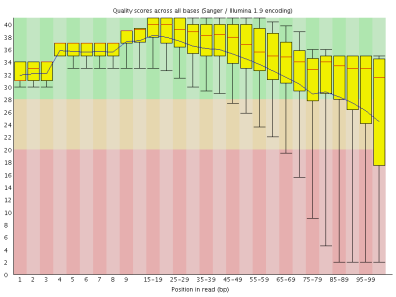
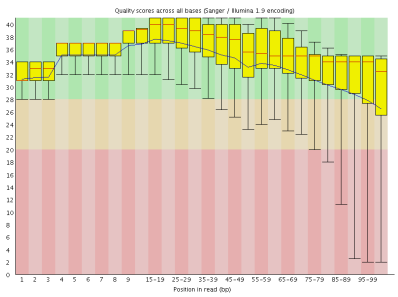
IWGSC_CSS_7AS_scaff_4245431	Kronos4485	3402	G	A	Hom	Hom	aaggcgcctggtgtttcC	aaggcgcctggtgtttcT	agtaagtggaAcagctaagatcaT
IWGSC_CSS_7BL_scaff_6667357	Kronos4485	641	C	T	Het	Het	gatcAgctgctcattcgagG	gatcAgctgctcattcgagA	ttccctgtcaattgatgccC

Appendix B

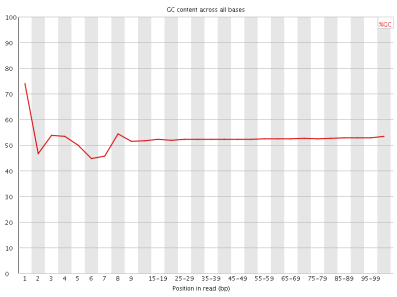
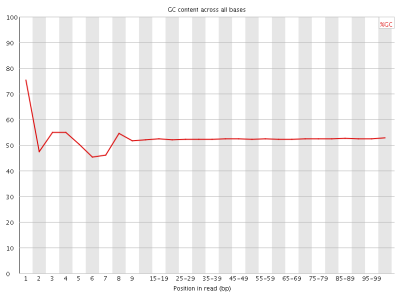
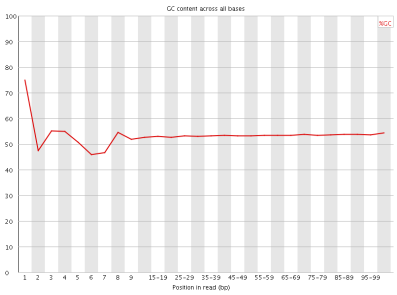
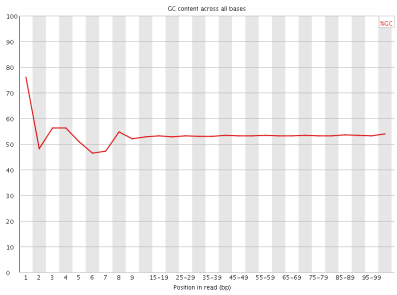
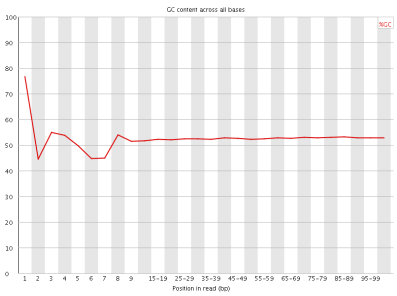
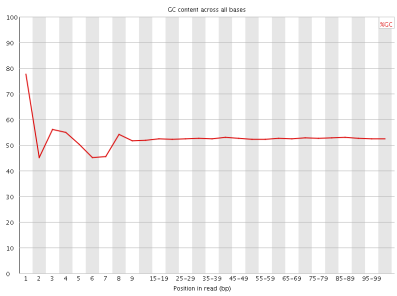
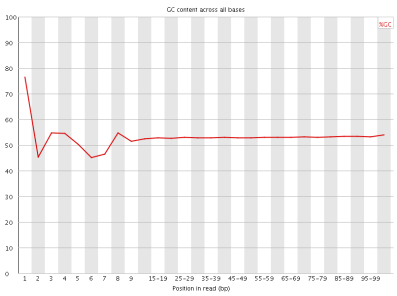
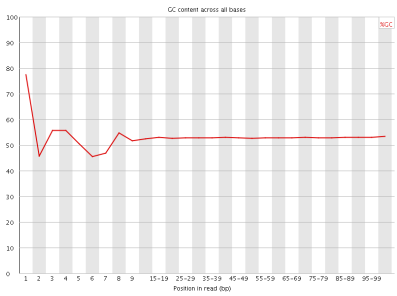
Quality Control

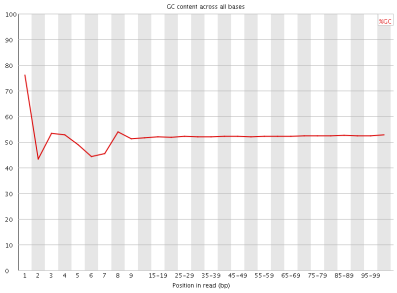
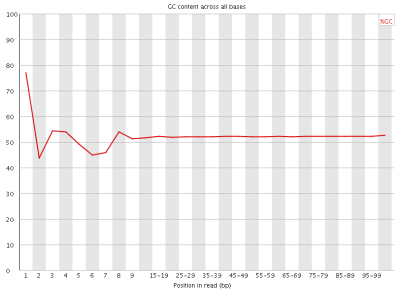
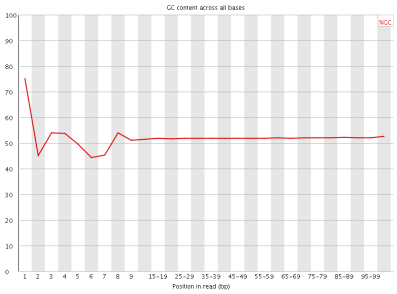
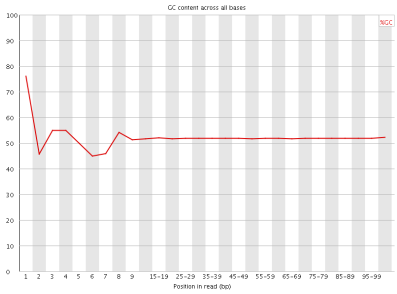
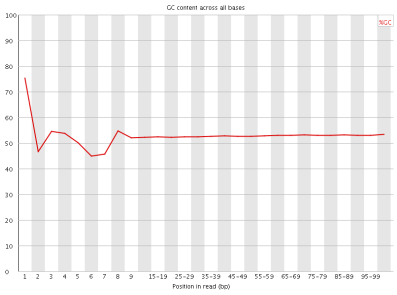
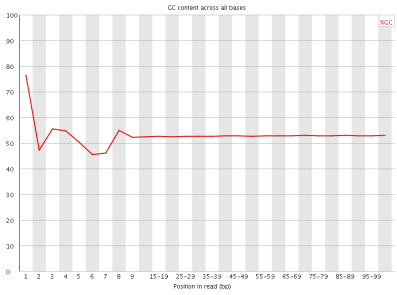
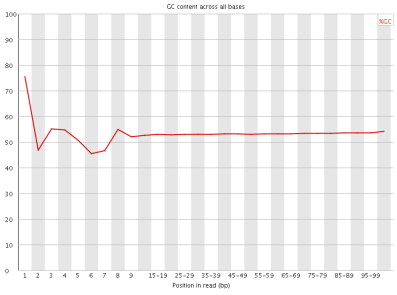
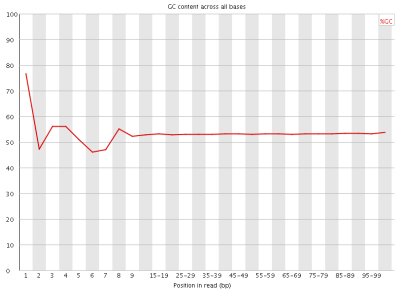
B.1 Sequence read quality

Sample	Read 1	Read 2
LIB1721 AvocetS		
LIB1722 AvocetS(Yr15)		
LIB1715 Bulk R1		

Sample	Read 1	Read 2
LIB1716 Bulk R2		
LIB1717 Bulk R3		
LIB1718 Bulk S1		
LIB1719 Bulk S2		
LIB1720 Bulk S3		

B.2 Sequence GC content

Sample	Read 1	Read 2
LIB1721 AvocetS		
LIB1722 AvocetS(Yr15)		
LIB1715 Bulk R1		
LIB1716 Bulk R2		

Sample	Read 1	Read 2
LIB1717 Bulk R3		
LIB1718 Bulk S1		
LIB1719 Bulk S2		
LIB1720 Bulk S3		

Appendix C

expVIP tutorial

AllConcat

Edit New Page

Ricardo H. Ramírez-Gonzalez edited this page a minute ago · 1 revision

Welcome to the expvip-web wiki! This wiki contains tutorials on how to setup the database and run it locally.

Updating the virtual machine Before loading the metadata, double click on `update_expvip.sh` in the desktop to get the latest version of expVIP.

1. Loading Virtual Machine. Instructions on how to setup Virtual Box to run expVIP
2. Loading Metadata. Detailed scripts in the virtual machine to prepare expVIP for your samples.
3. Loading data. Description on how to prepare and load the data to expVIP.
4. Running Kallisto. Instructions on how to run Kallisto and load the results in the database in a single step
5. Running Kallisto in batch. Instructions on how to run Kallisto and load the results in the database in a single step from multiple samples
6. Starting up the web server. Instructions on how to start the local web server for expVIP
7. Exporting Data. How to extract data from expVIP database.
8. Graphical Interface Tutorial. How to get the most of the expVIP graphical interface, exemplified with the Wheat Genome Browser.

Pages 11

Home
AllConcat
ExportData
List of tutorial videos
LoadingData
LoadingMetadata
LoadingVM
RunKallisto
RunKallistoBatch
StartWebServer
Tutorial expVIP Graphical Interface (Wheat Expression Browser example)

+ Add a custom sidebar

Clone this wiki locally

https://github.com/homonecloco/e

Clone in Desktop

Loading Virtual Machine

The `expVIP` Virtual Machine (VM) allows you to analyse your own RNA-Seq expression experiments locally.

Requirements

The virtual machine requires:

- VirtualBox, version 5 or newer.
- 6GB of RAM
- A 64-bit operating system running on an x86_64 architecture. (Intel, AMD)
- 10GB of free space.

Default data

The default values loaded in the virtual machine are available in this link. These correspond to the wheat data from Borrill, Ramirez-Gonzalez and Uauy, 2015 (*submitted*).

You can get a virtual machine with expVIP installed with either the wheat data preloaded or an empty database for your analysis here.

Available VMs

1. **expVIPNoData.ova** This VM is ready to use, but it has no data on it. You can load a custom set of RNA-seq reads, transcriptome reference and metadata.
2. **expVIPwithWheatData.ova** This has all the data loaded from www.wheat-expression.com. You can add your own data and compare it with the values of publicly available experiments.

Setup shared folders

To load your custom RNA-seq experiments, you have to setup a shared folder with your input files. This shared folder will contain the data and information required by the VM to implement expVIP and it provides the "connection" between your computer and the VM. This shared folder should include:

1. RNA-seq reads: as `fastq` or `fq.gz` files.
2. Transcriptome reference: currently only the cdna fasta file from ensembl is supported.
3. Metadata: this includes two separate files; one factor file and one metadata file (explained here).

Some important information:

- The shared folder must contain one sub-folder per each set of RNA-seq reads. So for example if you wish to analyse data from three samples, you will need three sub-folders (one each with the individual sample RNA-seq reads)
- Each RNA-seq sub-folder must be named with the same accession number that you use in your metadata (see here).
- If you wish to add your own wheat data to that previously provided in www.wheat-expression.com you will need to include sub-folders with your RNA-seq reads and then modify the metadata files: `default_metadata.txt` and `FactorOrder.tsv` which are provided in the `expVIPwithWheatData.ova` or can be downloaded here. Additional factors and metadata can be added at the end of these files following similar nomenclature as that already present in the files.

Loading the virtual machine

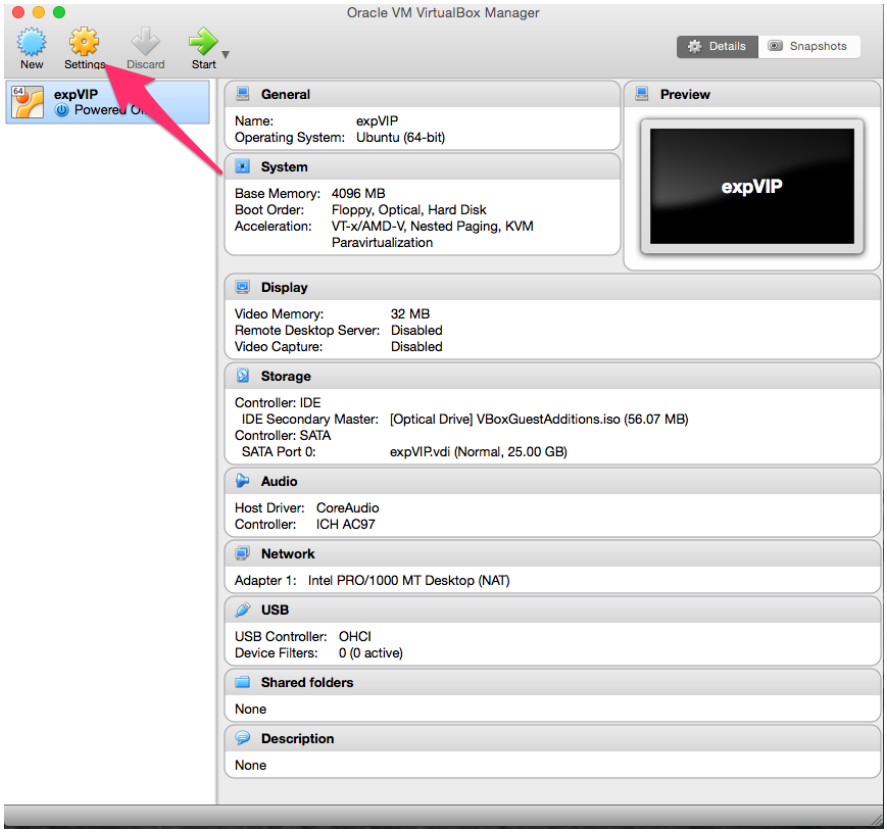
Download the `ova` virtual machine and double click it. Virtual Box will open it. Accept the default options.

If The virtual machine is not loaded, go to the menu `File` and click `Import appliance` . Open the `.ova` you want to use

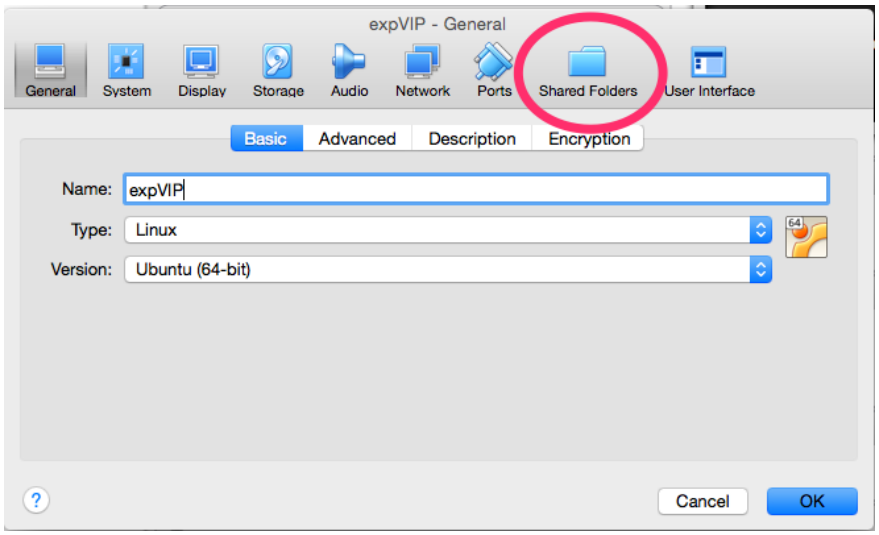
Availabe VMs:

- `expVIP.ova` expVIP is installed with an empty database. This VM requires to setup your own samples.
- `expVIPwithWheatData.ova` expVIP is installed with the wheat expression data, transcriptome reference and metadata. This VM allows the inclusion of additional samples to integrate with the previously analysed wheat data.

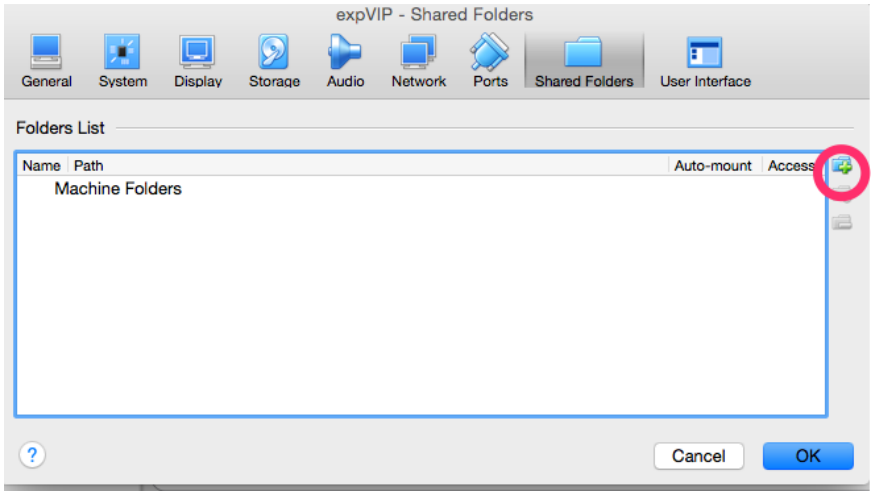
1. On the Oracle VM VirtualBox Manager select expVIP and click on the settings button



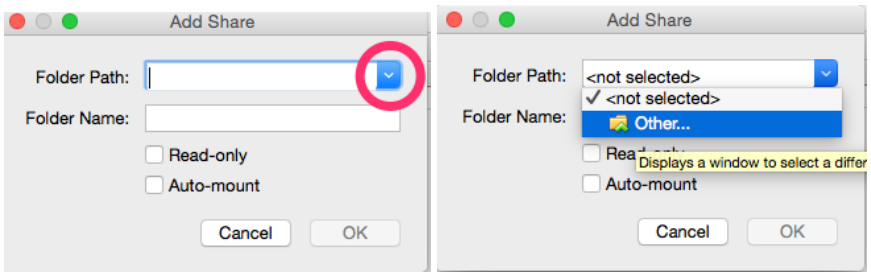
2. Click in `Shared folders`



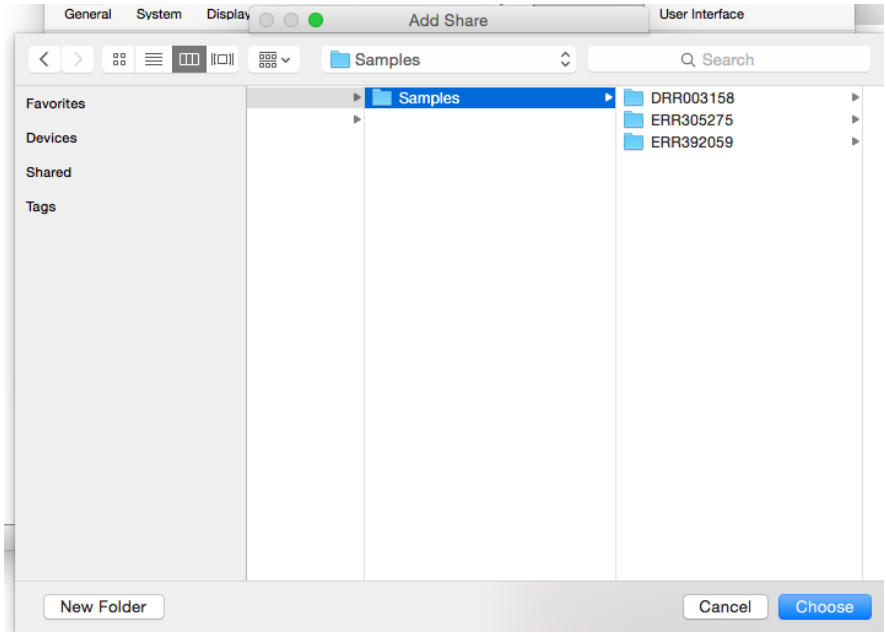
3. Add a new folder



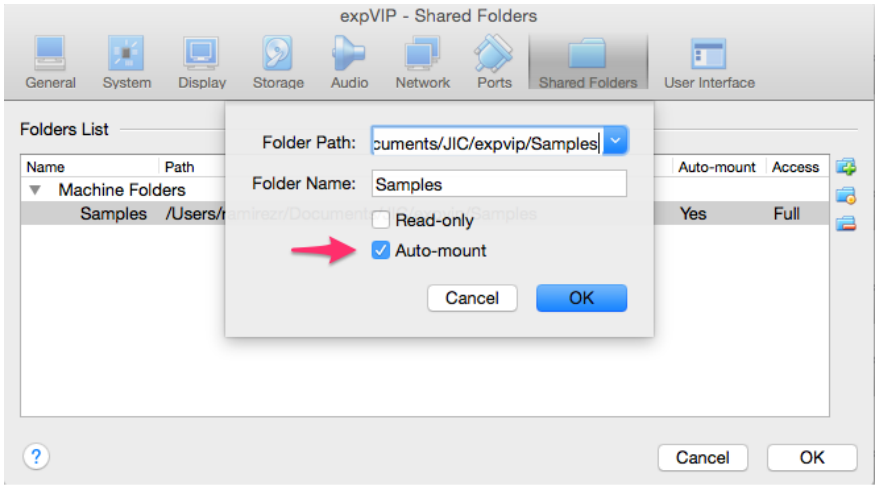
4. Search the `Folder path` with the experiments and the files with the metadata



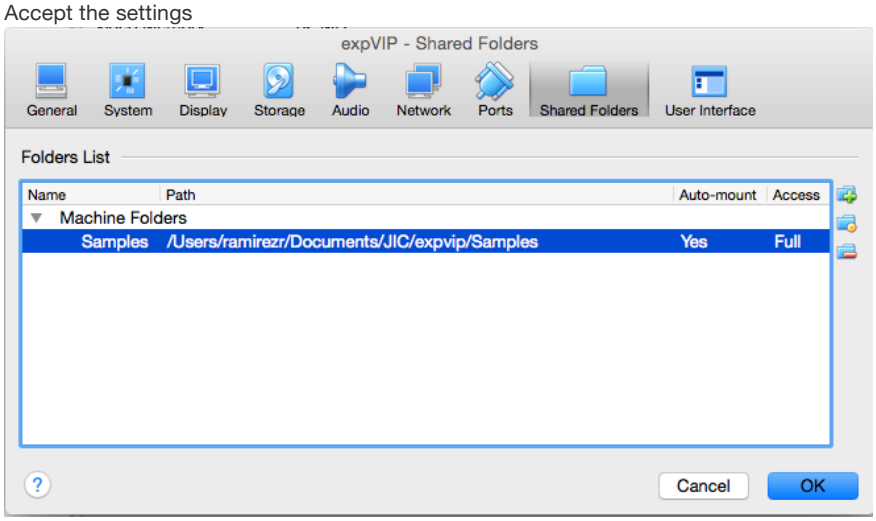
5. Select the folder



6. Make sure that the `Auto-mount` option is selected.

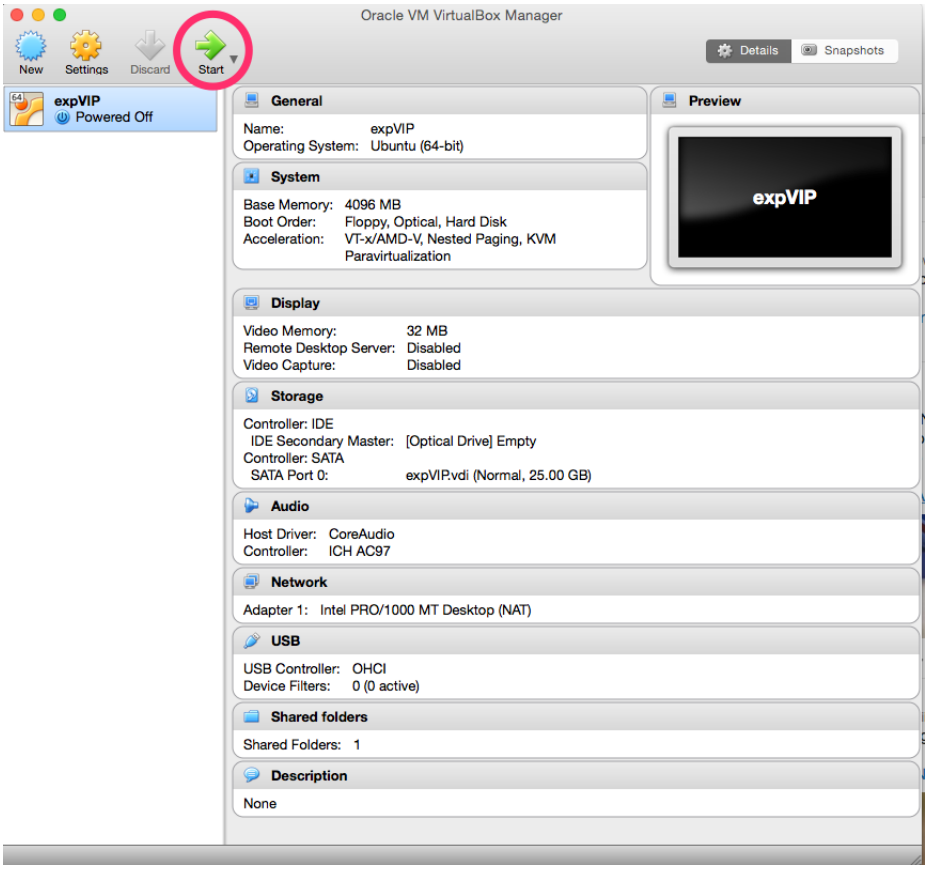


7. Accept the settings



Starting the virtual machine

Select `expVIP` from the VM list and press start.



NOTES

kallisto is included as part of the virtual machine and is free for non-commercial use. However, it requires a license for commercial use. The distribution of kallisto, with the corresponding license is included in `~/software/` in the VM.

Loading expVIP metadata

This tutorial covers the shell scripts that can be used to load the metadata with the graphical interface, with screenshots, and the rake task, to be run in the command line, if you are more comfortable in the terminal. The assumption is that expVIP is located in `~/expvip-web/`

Loading `factor file`

The first thing to do is to setup the available factors. The `factor file` is a text file, where each field is separated by tabs. A header is necesary on each column. The headers are the following:

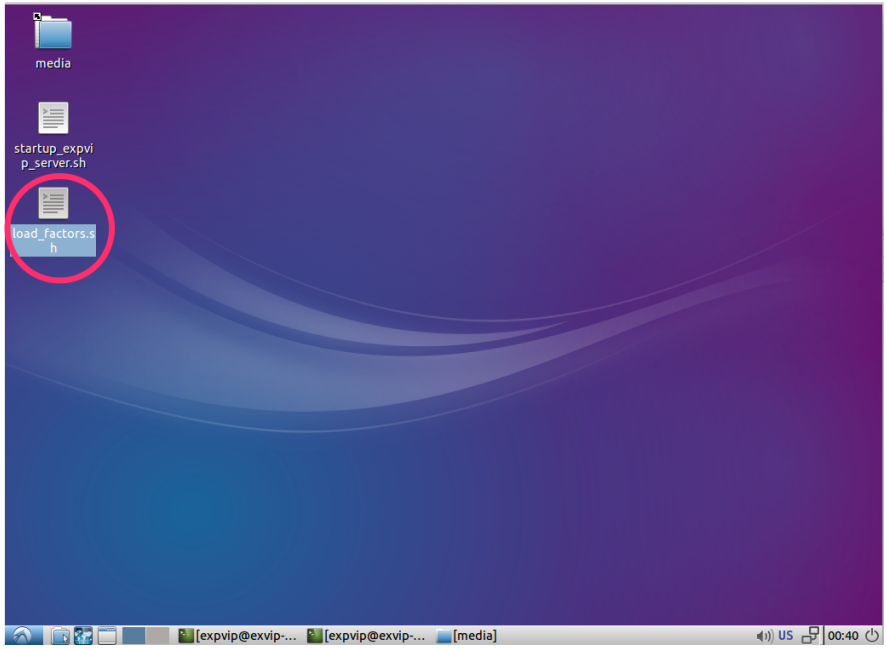
- **factor**: The name of the factor to group. These must match those used in the metadata file (see below).
- **order**: Default display order in the graphical interface.
- **name**: The long name of the grouped factor. These must match those used in the metadata file (see below).
- **short**: Short name of the grouped factor. This is used in the graphical interface when many factors are displayed.

`factor file` example:

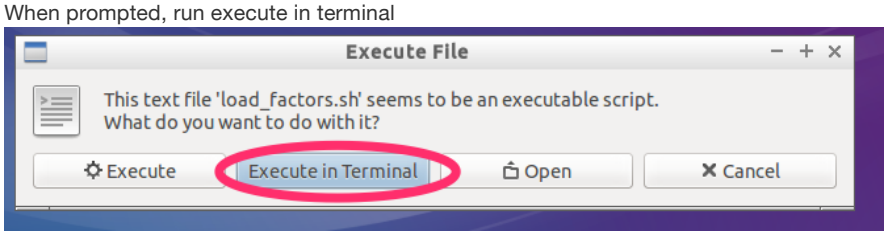
```
factor  order   name    short
Age 1   7 days  7d
Age 2   seedling stage  see
Age 3   14 days 14d
Age 4   three leaf stage    3_lea
Age 5   24 days 24d
Age 6   tillering stage till
Age 7   fifth leaf stage    5_lea
Age 8   1 cm spike  1_sp
Age 9   two nodes detectable    2_no
Age 10  flag leaf stage f_lea
Age 11  anthesis    anth
Age 12  2 dpa   2dpa
Age 13  4 dpa   4dpa
High level age  1   seedling    see
High level age  2   vegetative  veg
High level age  3   reproductive    repr
High level stress-disease   1   none    none
High level stress-disease   2   disease dis
High level stress-disease   3   abiotic abio
High level stress-disease   4   transgenic  trans
High level tissue   1   spike   spike
High level tissue   2   grain   grain
...
```

Wizard to load factors

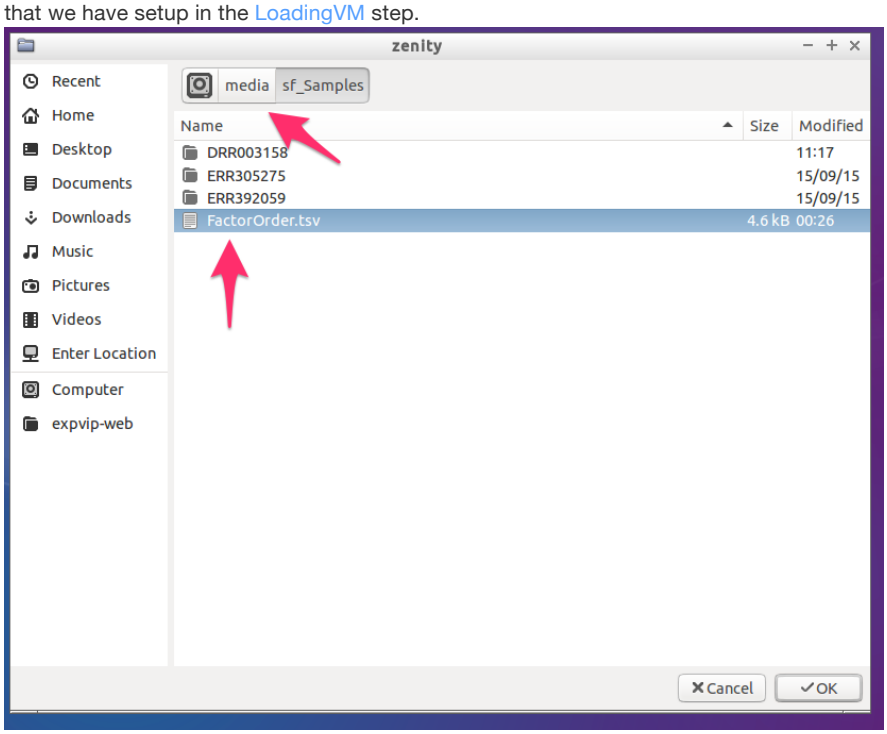
1. Double click in `load_factors.sh` in the desktop



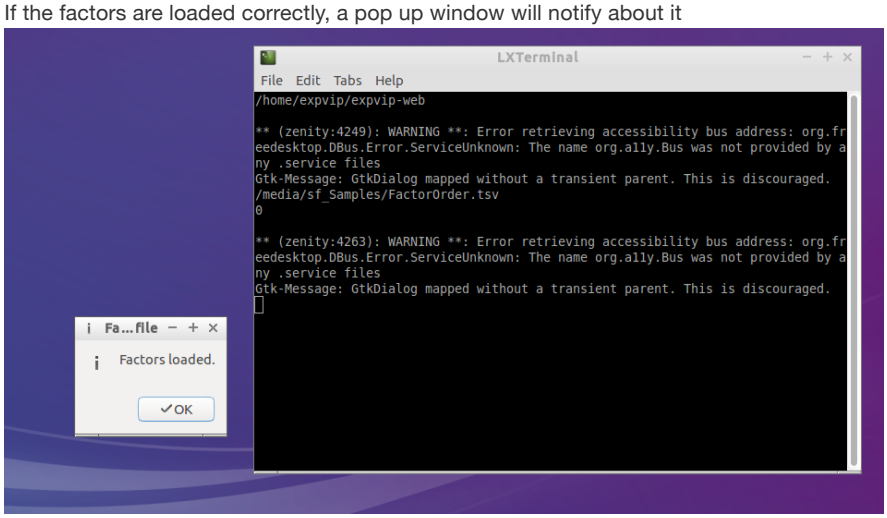
2. When prompted, run execute in terminal



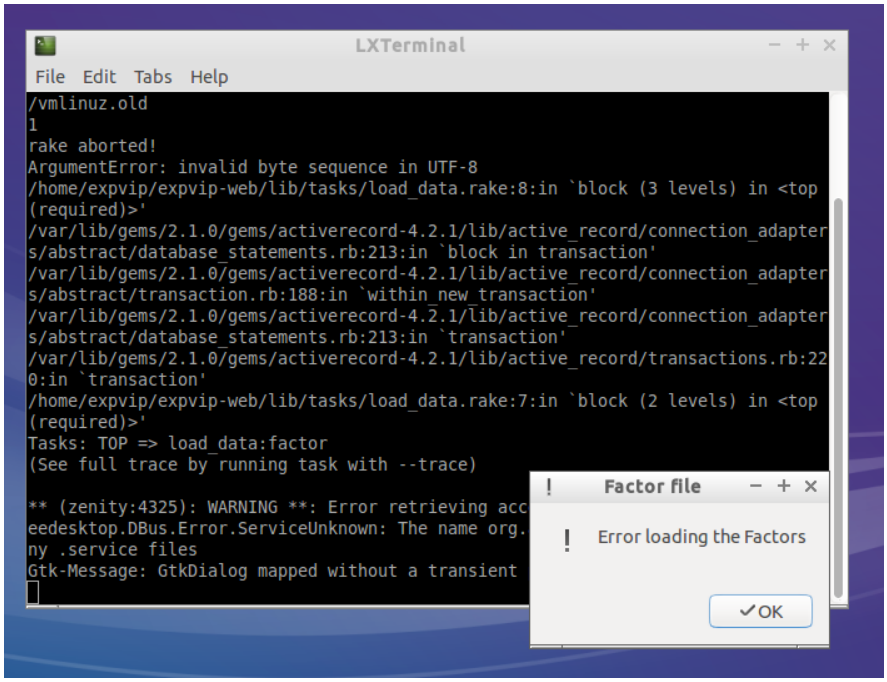
3. By default, the script goes to `/media`, which is the folder containing the `shared folders` that we have setup in the LoadingVM step.



4. If the factors are loaded correctly, a pop up window will notify about it



5. If there was an error loading the factors, a message will notify about it. The error log may give a hint of what went wrong, but if you can't figure out send a screenshot of the terminal to the developers.



Rake task

To load the factors, you can run directly the rake task from `~/expvip-web/` .

```
rake load_data:factor[FILE_WITH_FACTORS];
```

Loading metadata

The second step is to load the experiment metadata. Currently, a tab separated file is the input and it **must** contain the following columns with the header named exactly as stated:

- **secondary_study_accession**: The accession number for experiments carried as part of a single study. This is usually the high level BioProject or SRA number.
- **run_accession**: The accession of the individual run.
- **scientific_name**: of the species.
- **experiment_title**: A description for the individual RNA-seq sample.
- **study_title**: A description of the general study.
- **Variety**
- **Tissue**
- **Age**
- **Stress-disease**
- **Manuscript**: The DOI of the study.
- **Group_for_averaging** A description of the experiment. This must be the same all the replicates in the same study.
- **Group_number_for_averaging**: A short name for replicated experiments.
- **Total reads**: (optional)
- **Mapped reads**: (optional)
- **High level variety**: A higher level grouping to get summarized data of the factors.
- **High level tissue**
- **High level age**

- **High level stress-disease**

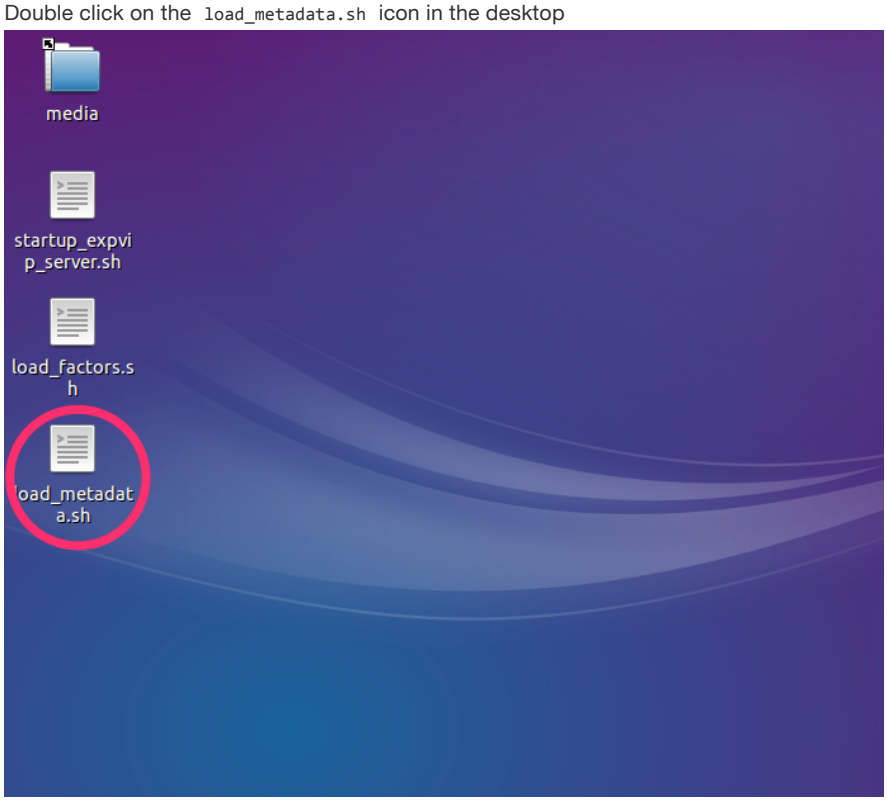
Important points

- `Variety` , `Tissue` , `Age` , `Stress-disease` , and their corresponding `High level` factors must be exactly the same as in the columns `factor` and `name` from the `factor file` (see above).
- The graphical interface will group samples based on these factors. Therefore these can be defined based on the user needs. For example the factor `High level tissue` will include tissue types such as `grain` , `roots` , `spike` and `leaves/shoots` . Within each of these tissue types, a more detailed description can be included under the `Tissue` heading. For example: `starchy endosperm` , `seed coat` , `transfer cells` , etc. RNA-seq samples which share factor names in common will be displayed as groups in the visual interface.
- If `Mapped reads` and `Total reads` are missing, you need to run `kallisto` mapping from the `rake` task.

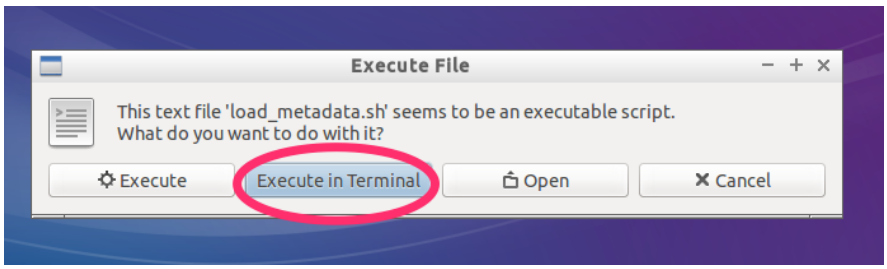
Using the graphical interface

The process is similar to loading the factors. However, the metadata file is selected.

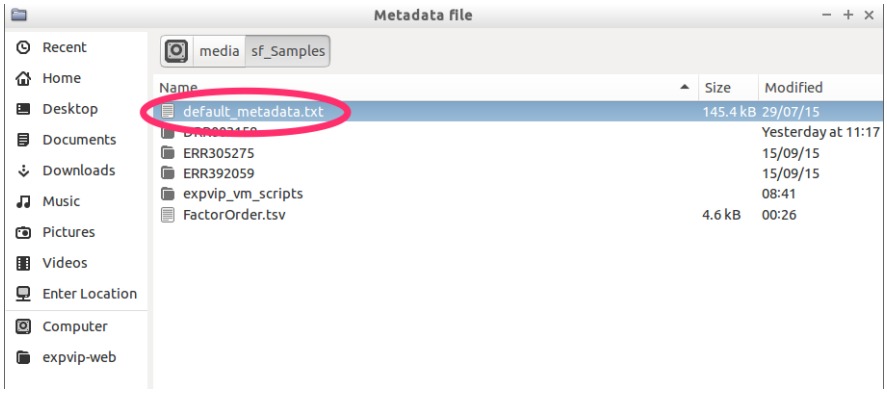
1. Double click on the `load_metadata.sh` icon in the desktop



2. Choose execute in terminal



3. Select the metadata file



Rake task

```
rake load_data:metadata[FILE_WITH_THE_METADATA]
```

Loading the gene sets

Before loading the actual expression data or running kallisto, it is necessary to load the gene models. Currently, only the fasta file with the cdna from ensembl is supported. The fasta header should contain the following fields, besides the gene name (first string in the header).

- **cdna**
- **chromosome** or **scaffold** are converted to position
- **gene**
- **transcript**
- **description** a free text, in quotes. Any other field with quotes may fail in the load.

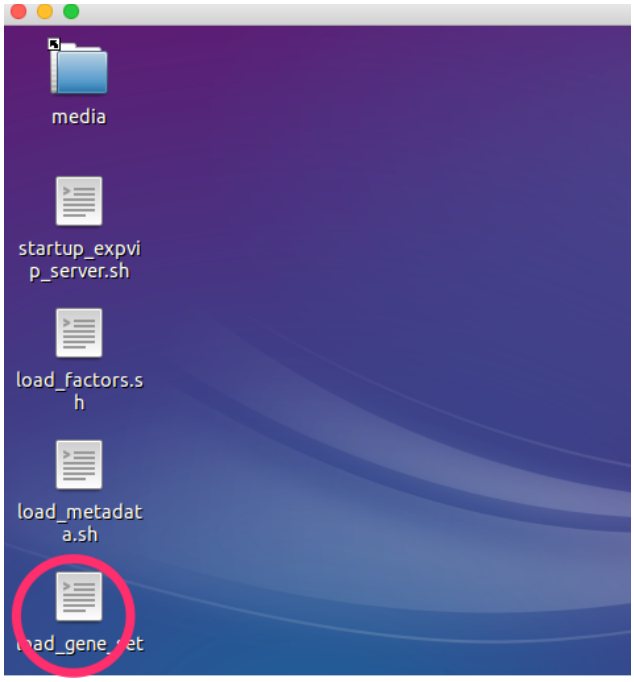
Besides the fasta file, it is necessary to give a name to the gene set. For this tutorial, the `gene_set` will be `IWGSC2.26`

Example fasta file

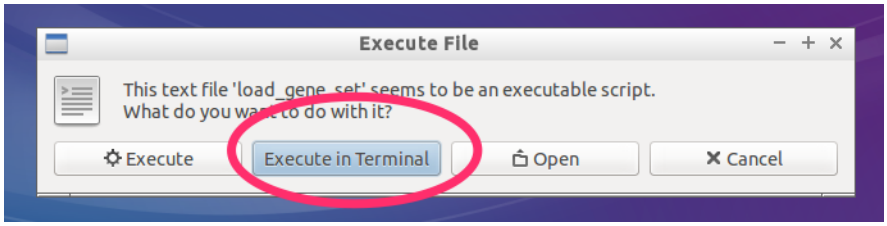
```
>Traes_5BL_3FC5BA305.1 cdna:novel scaffold:IWGSC2:IWGSC_CSS_5BL_scaff_1082268:5:199:-1 ge
TGCTGCTGCTAGGCTTGAAGAGGTTGCTGGCAAGCTCCAGTCTGCTCGGCAGCTCATTCA
GAGGGGCTGTGAGGAGTGCCCCAAGAACGAGGATGTTTGGTTCGAGGCATGCCGGTTGGC
TAGCCCAGATGAGTCAAAGGCAGTAATTGCCAGGGGTGTGAAGGCAATTCCCAACTCTGT
GAAGCTGTGGCTGCA
>Traes_6BL_9BB648D51.1 cdna:novel scaffold:IWGSC2:IWGSC_CSS_6BL_scaff_430516:302:1741:-1
TCCCTATCTGTTTCCTTGGCAGCTCCCTGATCCAATCGATCCATCAGGGCTCGACTAACT
TCTTCCAGCGCCTCTTCAGCGCGGGAGATCTACCAGCGTCGGCGGAGGGGCGTAGGTGCA
GGCGTGCAGCCCAAGTCCGCACCCGGCTCTAGGTTTCTGCTAATCTTCTTCCACCTGTGA
TACGCGCTCCGGGGCTAGGAGCACTCGTTGCCGGCTGCCTCGTGCTCGGAATGGCGGATG
```

Graphical interface

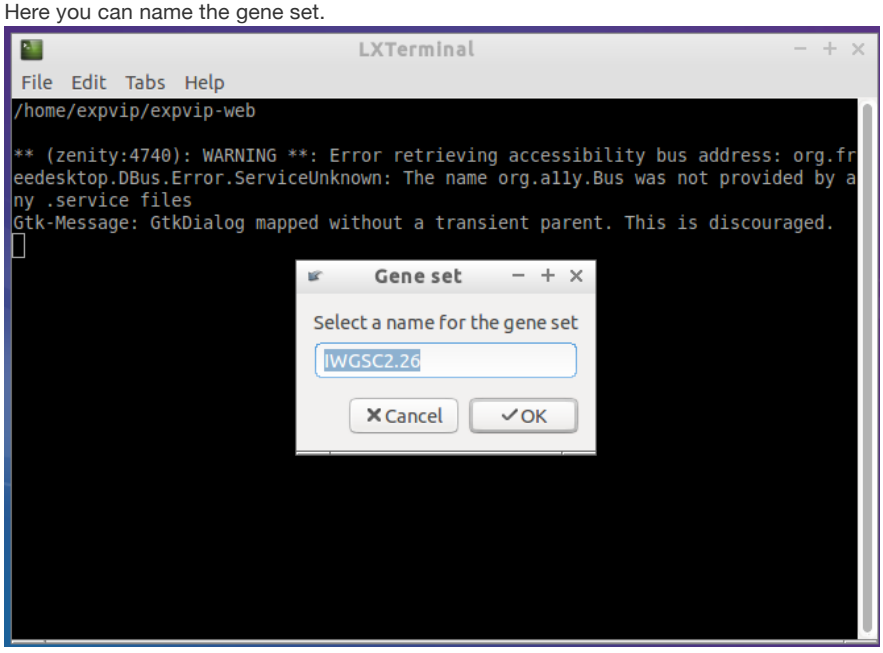
1. Double click on the `load_gene_set` script



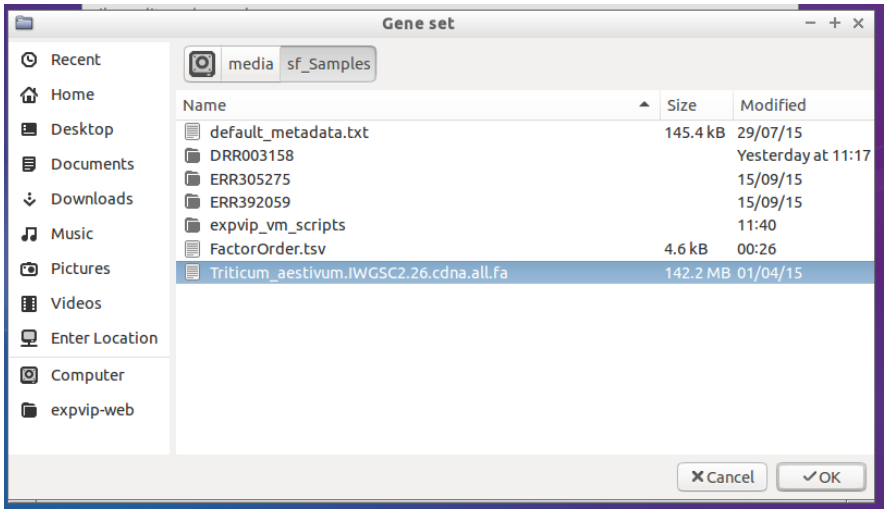
2. Select `Execute in Terminal`



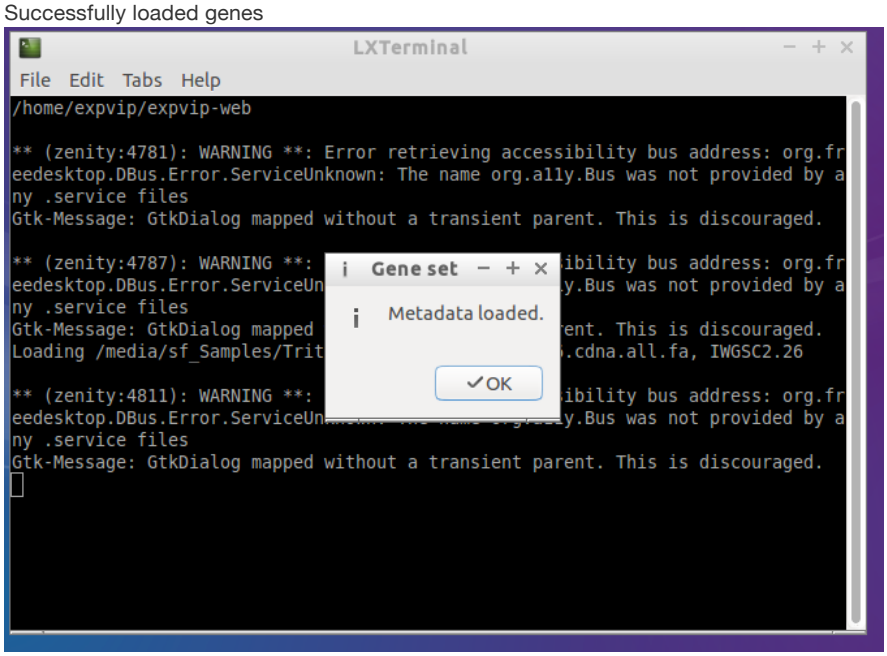
3. Here you can name the gene set.



4. And select the reference file. This may take a few minutes to load.



5. Successfully loaded genes



Rake task

```
rake load_data:ensembl_genes[IWGSC2.26,/Triticum_aestivum.IWGSC2.26.cdna.all.fa]
```

Loading the homoeologues

In order to show the homoeologues, a file with the homoeologies must be loaded. The file is tab separated with the following format:

```
Gene    A   B   D   Group   Genome
Traes_5BS_0AFC3F795     Traes_5BS_0AFC3F795 Traes_5DS_C204EBAA9 5   B
Traes_5DS_C204EBAA9     Traes_5BS_0AFC3F795 Traes_5DS_C204EBAA9 5   D
Traes_7DL_82360D4EE1            Traes_7DL_82360D4EE1    7   D
Traes_2AL_1368BE0AD Traes_2AL_1368BE0AD Traes_2BL_CD459994C1        2   A
...
```

Note that the gene names are not the same as the transcript names, they correspond to the gene name.

Generating the file with the homoeologues from Ensembl compara

The file can be genrated with ensembl compara, using the following query:

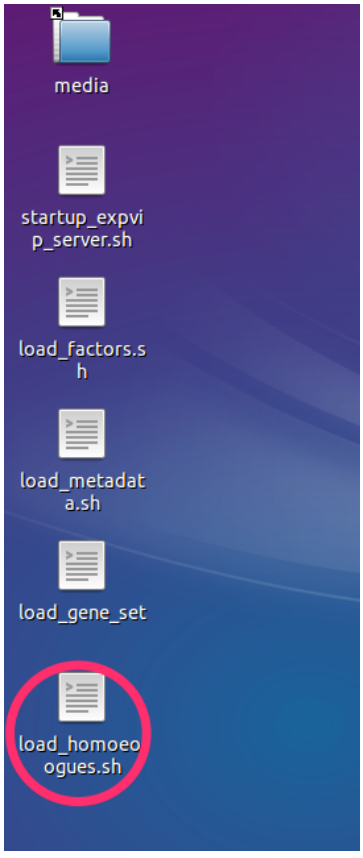
```
SELECT
    homology_member.homology_id, cigar_line, perc_cov, perc_id, perc_pos,
    gene_member.stable_id as genes,
    gene_member.genome_db_id

FROM
    homology_member
INNER JOIN homology USING (homology_id)
INNER JOIN method_link_species_set USING (method_link_species_set_id)
INNER JOIN gene_member USING (gene_member_id)
WHERE method_link_species_set.name="T.aes homoeologues";
```

Then, to format the result of the query (saved as `compara_homology.txt`), you can use the provided script

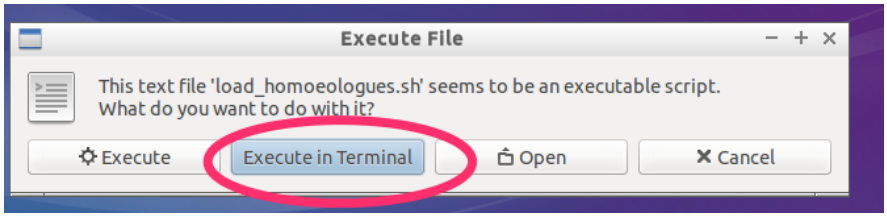
```
ruby bin/homologyTable.rb compara_homolgy.txt homology.txt homology_counts.txt
```

You can get your homoeologies elsewhere, as long as you keep the file format.

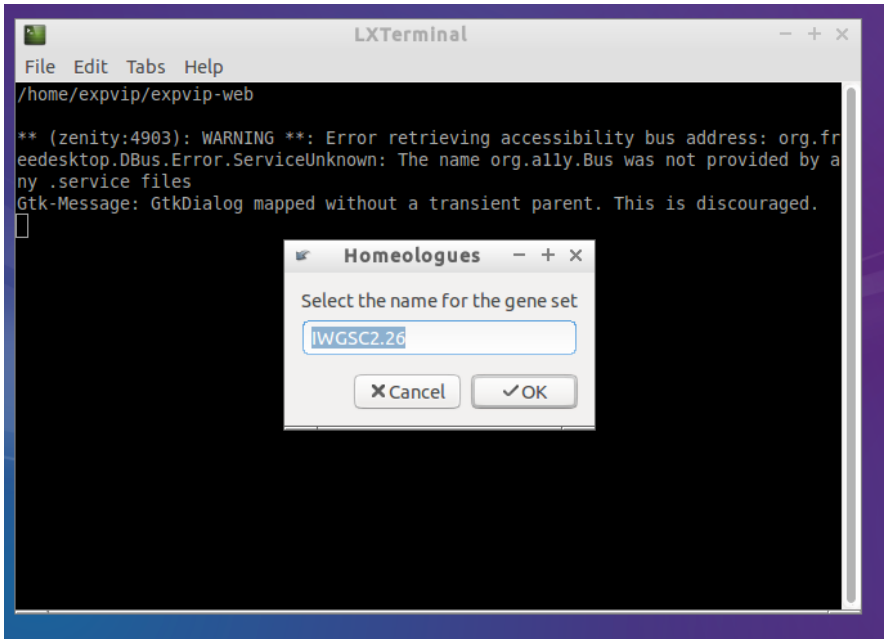
At this point, the homoloeologues are called A,B and D. This is going to change on a future release to allow any chromosome group naming.



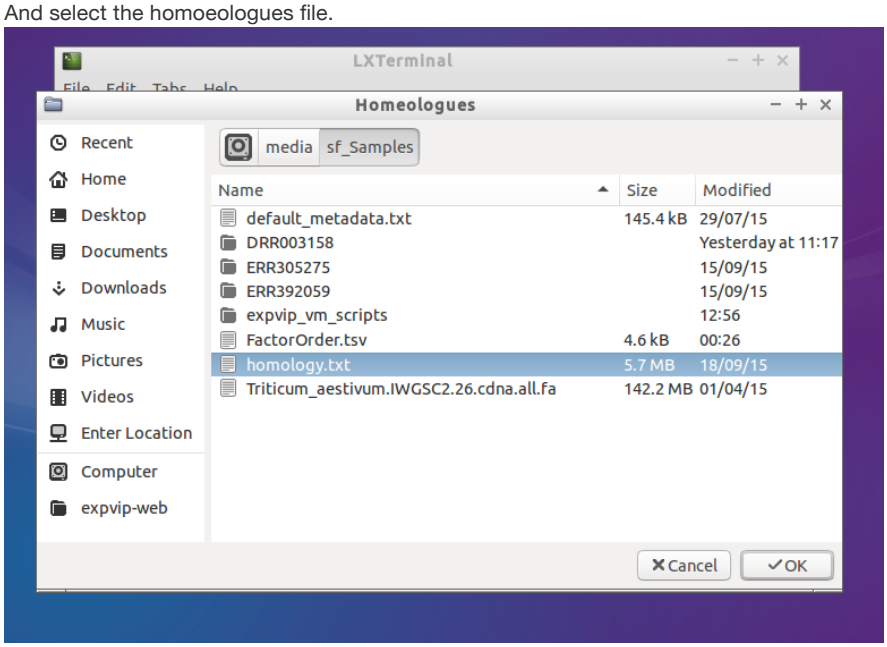
1. Double click on the `load_homoeologues.sh` script
2. Select `Execute in Terminal`



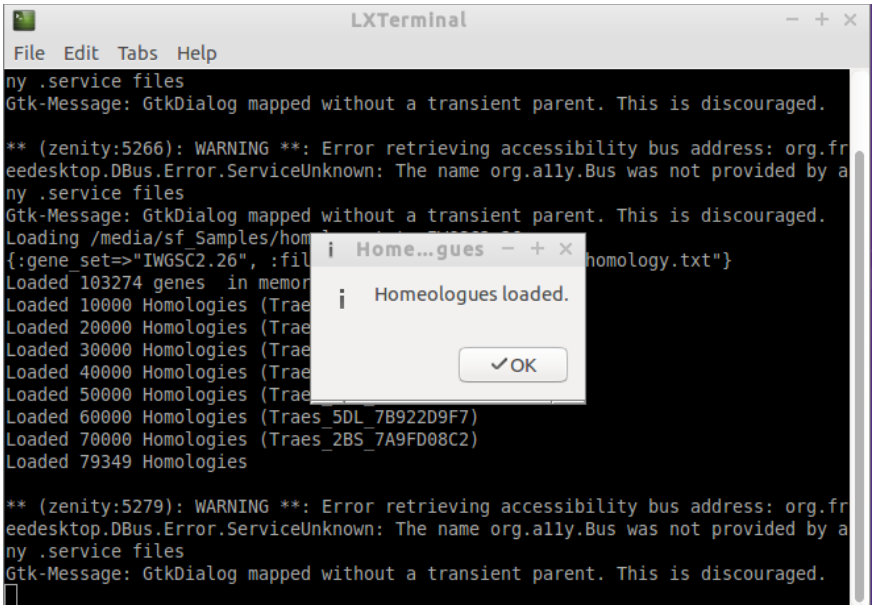
3. Here you can name the gene set. It must be the same name you added for the gene reference.



4. And select the homoeologues file.



5. Succesfully loaded. At the end of the log you can see which how many homologies where loaded.



Rake task

```
rake load_data:homology[IWGSC2.26,/homology.txt]
```

Data loading

Once the database has been created, expVIP currently supports two methods to load expression data onto the database:

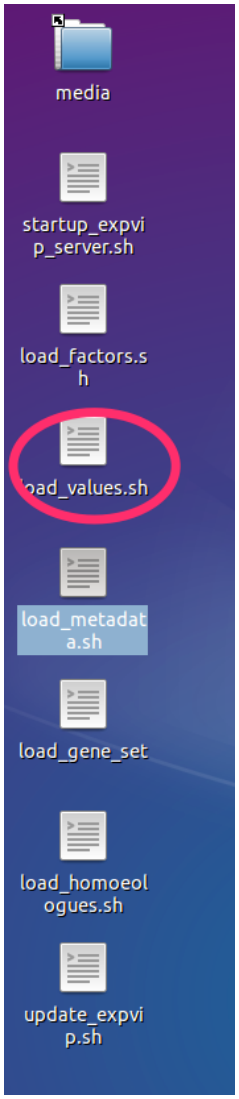
1. Load the precomputed expression values into the database, or
2. Run kallisto to generate the expression data. These are then loaded directly into the database.

Single big table

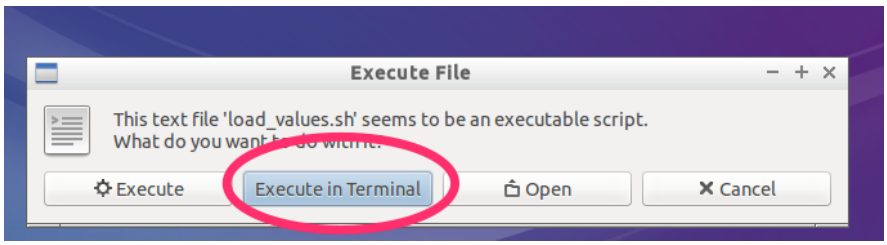
The fastest way to load the data to expVIP is to produce a table with all the values for each expression unit (tpm, counts). The table must contain a column `target_id` that has the gene name, as the first field in the fasta file used for the mapping. The rest of the columns most contain a header with the accession of the experiment. Each row represents a value. All the values in the table must be from the same time.

For the case of wheat in which we have already generated the kallisto mapping of 418 RNA-seq studies, the expression table can be downloaded directly from here. The `txt` files are called `final_output_counts.txt` and `final_output_tpm.txt` for the corresponding expression unit.

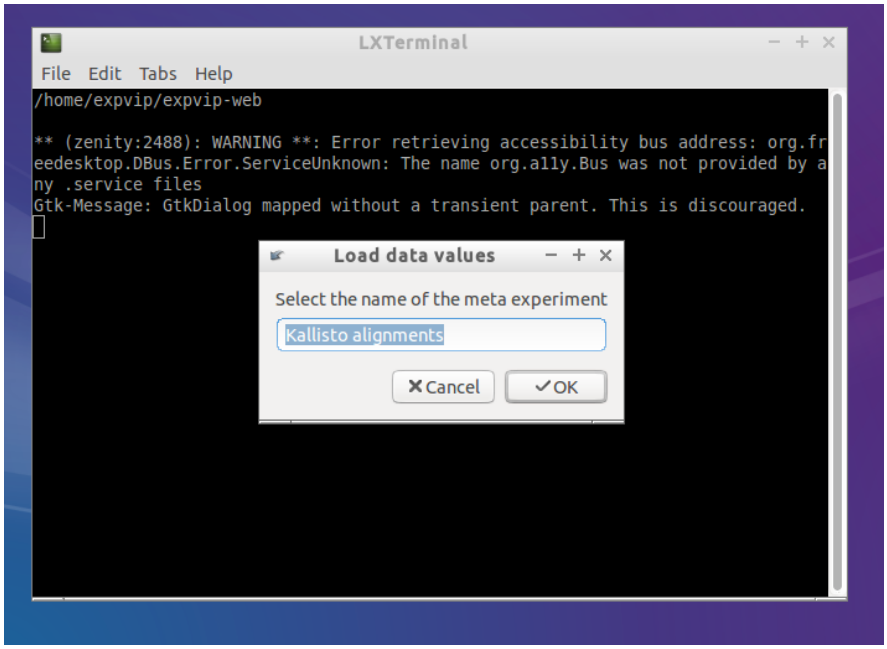
Loading from the script



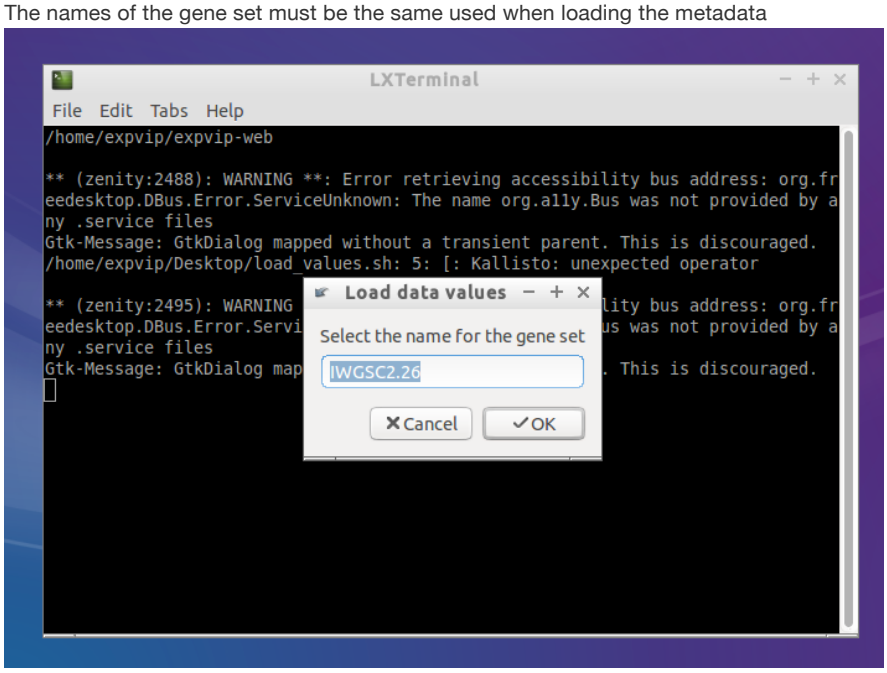
1. Double click on the `load_values.sh`
2. Click one `Execute in Terminal`



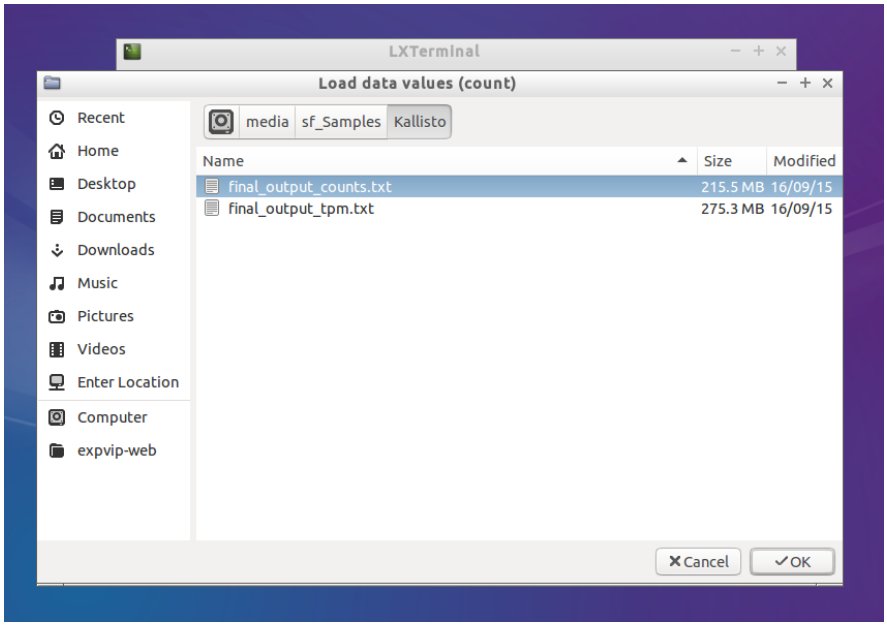
3. Select a name for the set of alignments. expVIP can keep several runs of alignments in the database. The ability to select between them will be added in a future release.



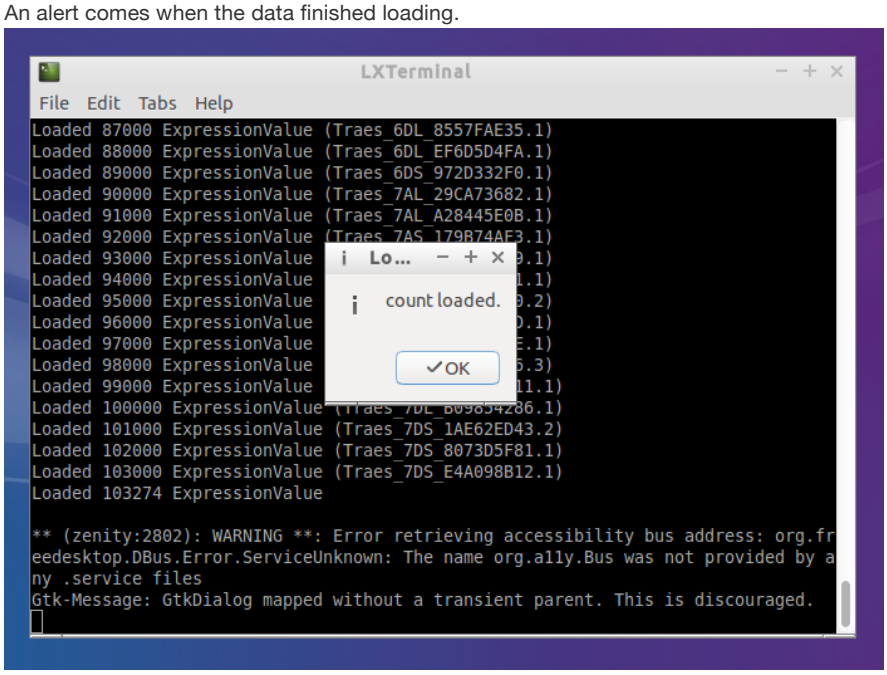
4. The names of the gene set must be the same used when loading the metadata



5. Select the file with the big table. The process takes some time, so be patient.



6. An alert comes when the data finished loading.



If the accession numbers are not the same as in the metadata the process will fail.

Rake Task

In order to load the data, the task `load_data:values` is provided. For example, to load the tpm, the following command is used.

```
rake "load_data:values[First run,IWGSC2.26,tpm,edited_final_output_tpm.txt]"
```

Running Kallisto

You can load the data directly to the database provided that you generated the `Kallisto`

index on your reference:

```
kallisto index --index=Triticum_aestivum.IWGSC2.26.cdna.all.fa.kallisto.k31 Triticum_aest
```

You can modify the index options as you find it suitable for your experiment.

To run Kallisto on single sample, the following task is available:

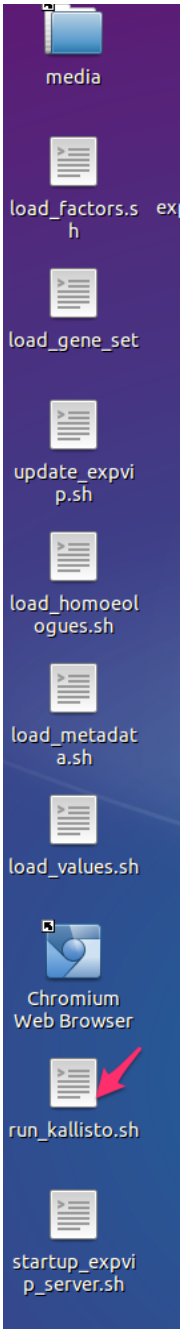
```
rake kallisto:runAndStorePaired[Index,folder/with/samples/ACCESSION,experiment_title,IWGS
```

The task requires that the reads are in a folder named exactly as the `secondary\_study\_accession*` column in the metadata file. If the accession doesn't exist, the task will fail. `experiment_title` is a name to group alignments.

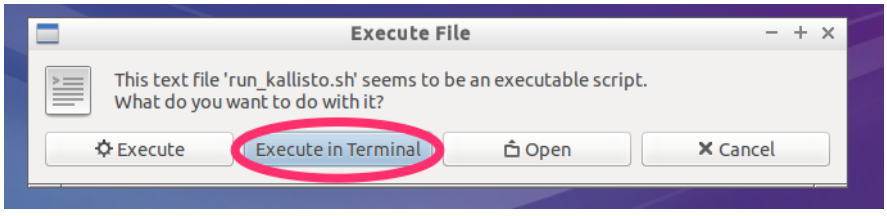
Run Kallisto on a single sample

expVIP can run `Kallisto` and load the `tpm` and `counts` to the database. The only requirement is to run `kallisto index` on the transcriptome reference.

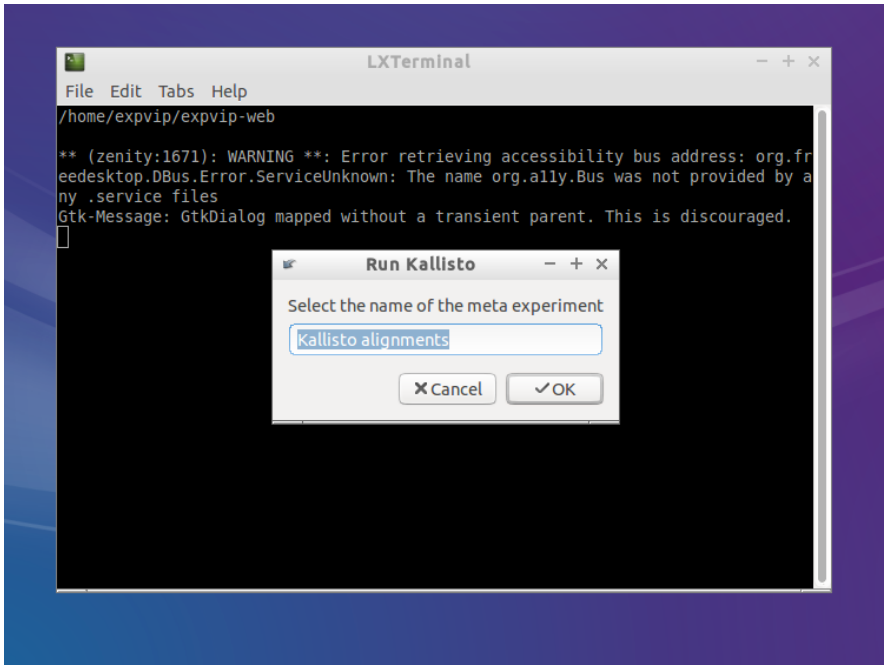
Graphical interface



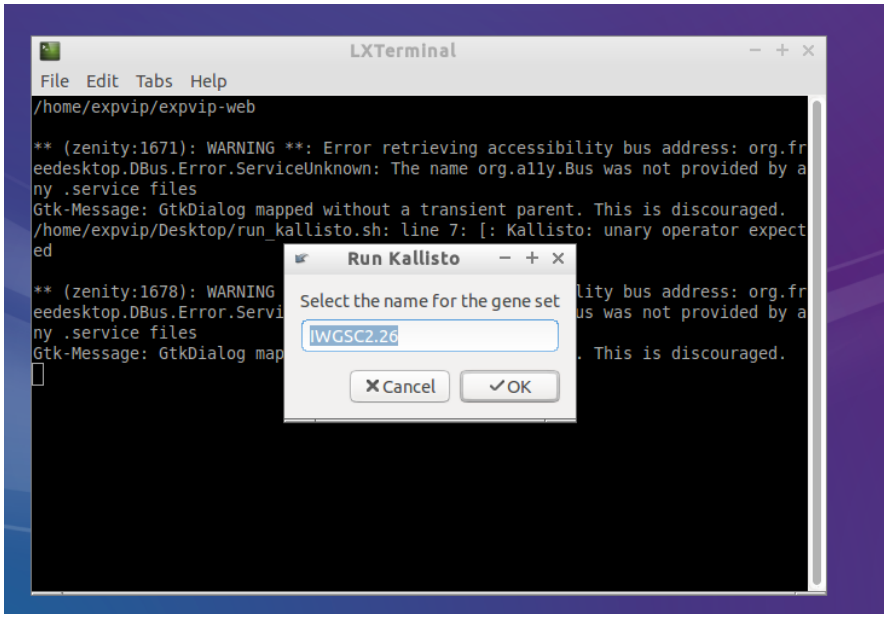
1. Double click on `run_kallisto.sh`
2. Click on `Execute on terminal`



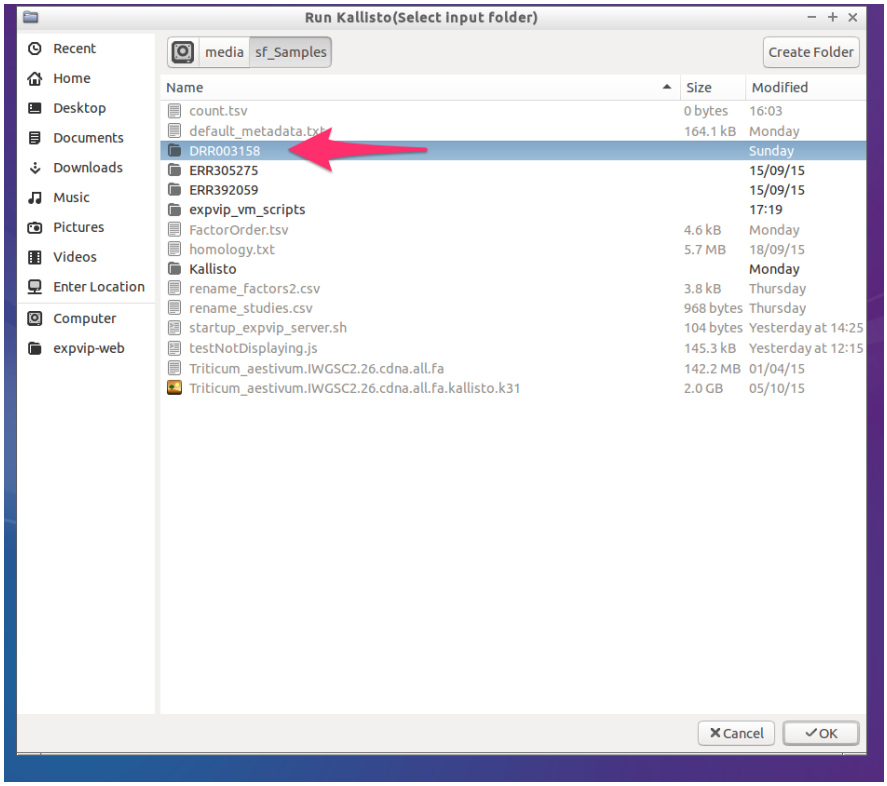
3. Give a name to the set of mappings to be grouped. All mappings done with the same reference and preference should have the same name.



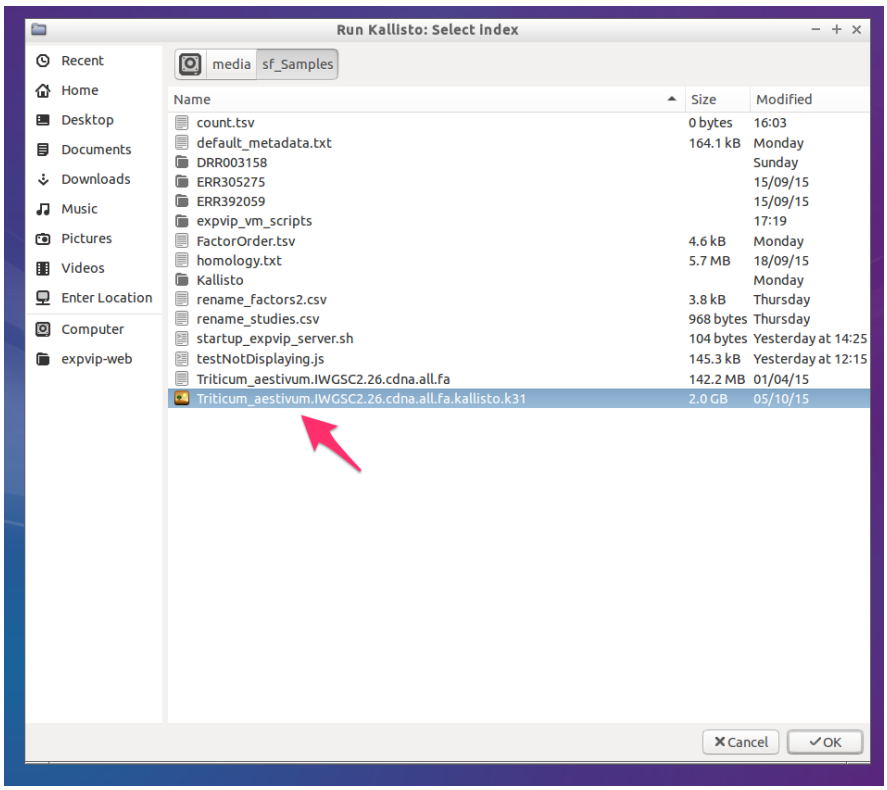
4. Get the name of the reference. This name must be the same used when loading the metadata



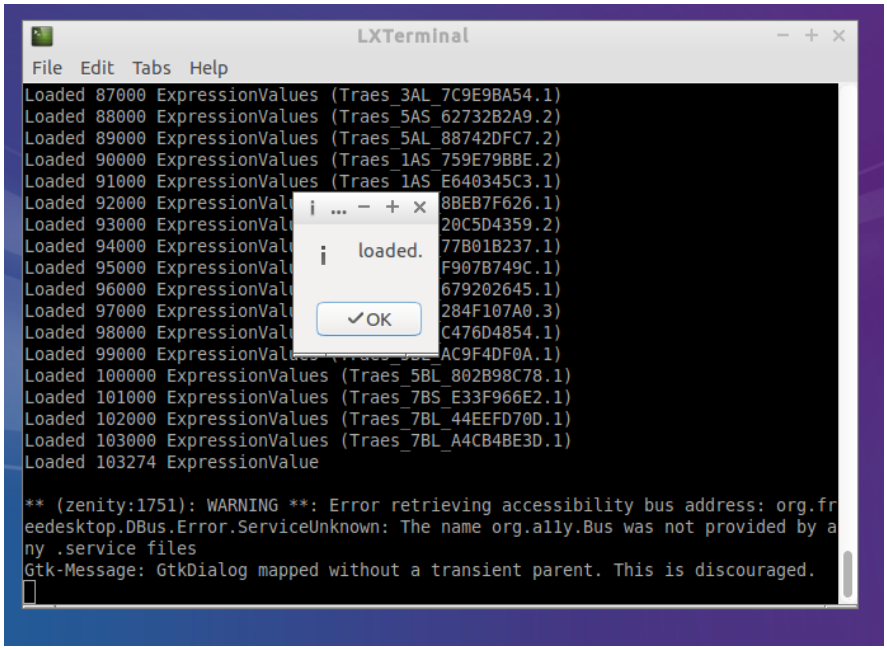
5. Select a folder with the reads. The reads must be paired reads. The folder name must be the same as the `accession` used on the metadata.



6. Select the kallisto index



7. Wait for Kallisto to run and load the data



You can reepeat this with all the samples or you can use the batch load.

Rake task

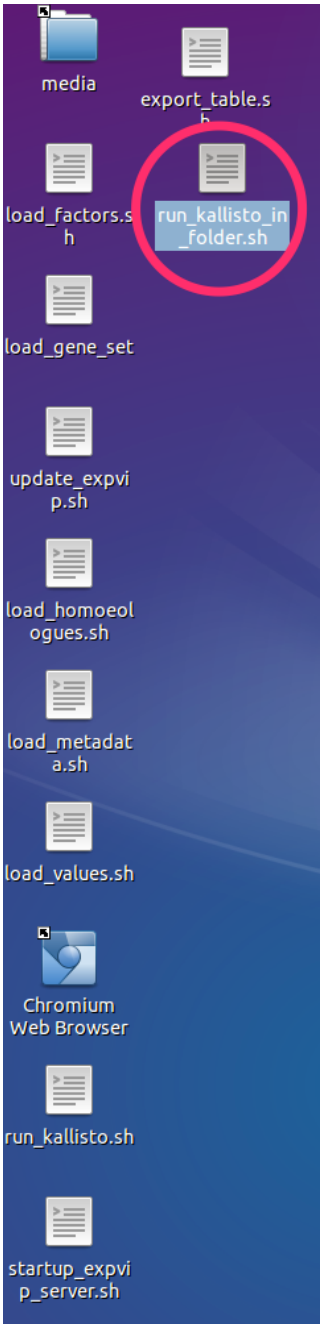
```
kallisto:runAndStorePaired[kallistoIndex,input_folder,metaExperimentName,geneSetName]
```

Where `metaExperimentName` is the name of the group of alignments under the same conditions and ` `geneSetName` is the name of the reference.

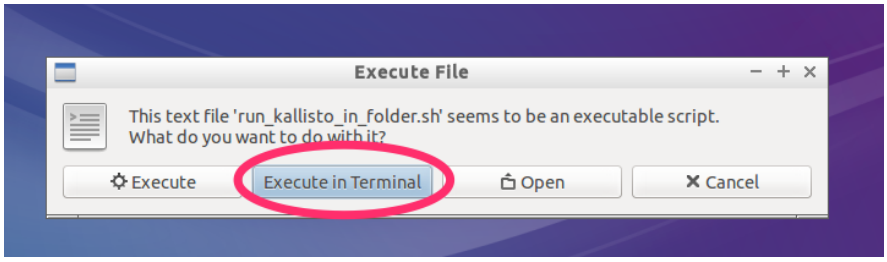
Run Kallisto on a multiple samples

expVIP can run `Kallisto` and load the `tpm` and `counts` to the database from multiple samples. The only requirement is to run `kallisto index` on the transcriptome reference.

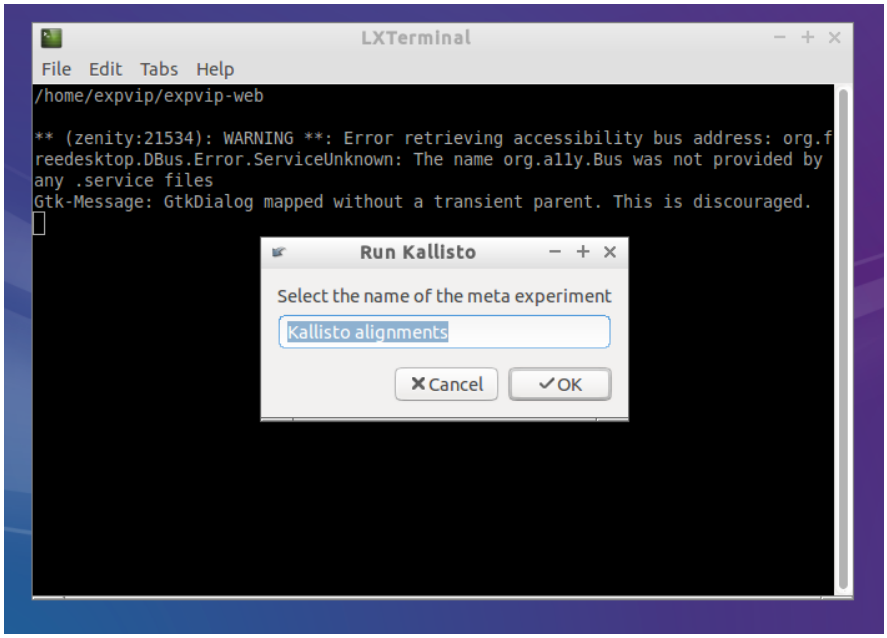
Graphical interface



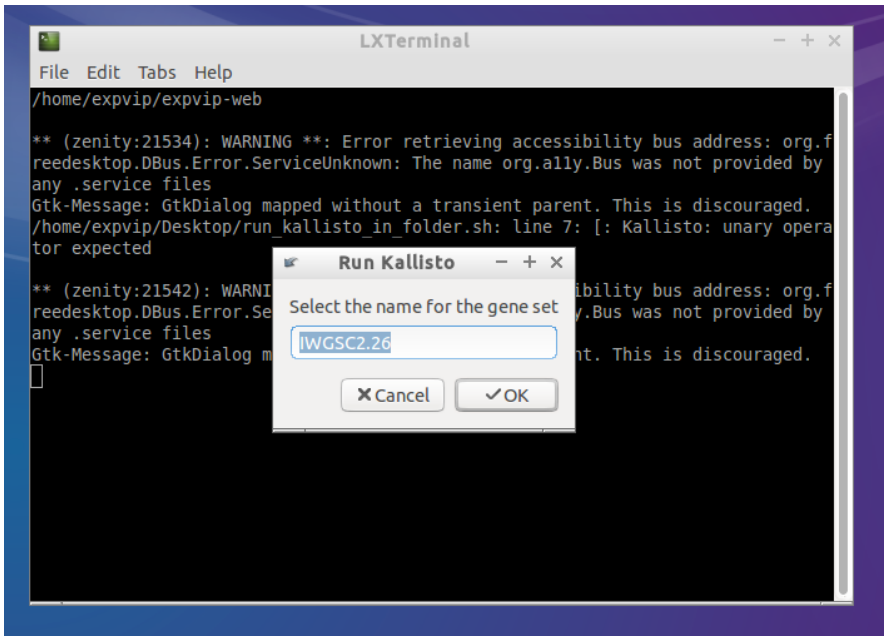
1. Double click on `run_kallisto.sh`
2. Click on `Execute on terminal`



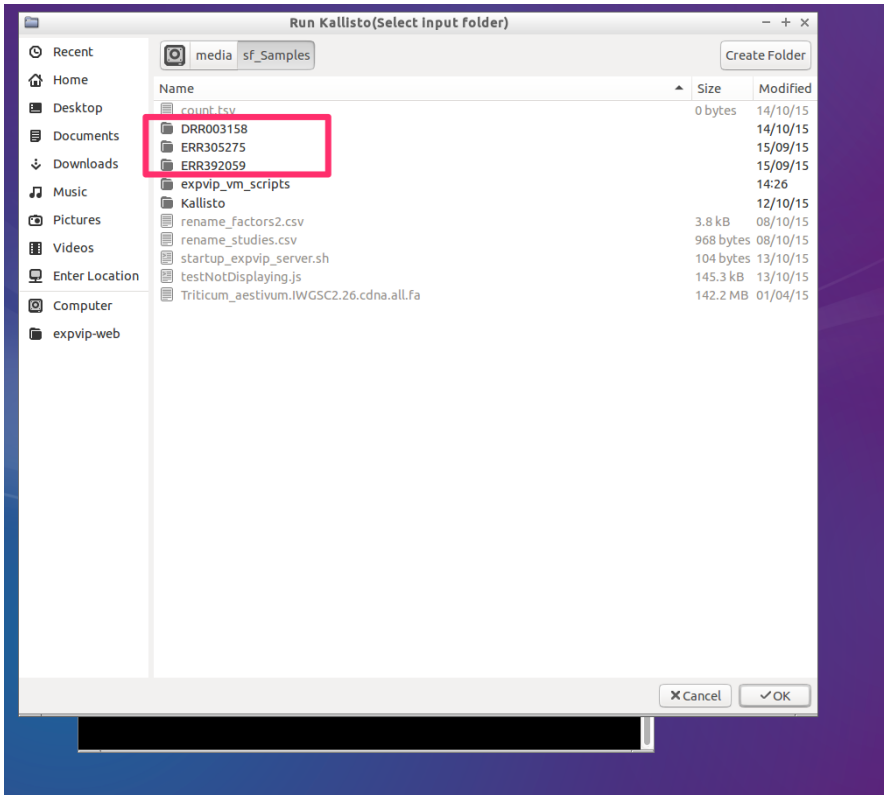
3. Give a name to the set of mappings to be grouped. All mappings done with the same reference and preference should have the same name.



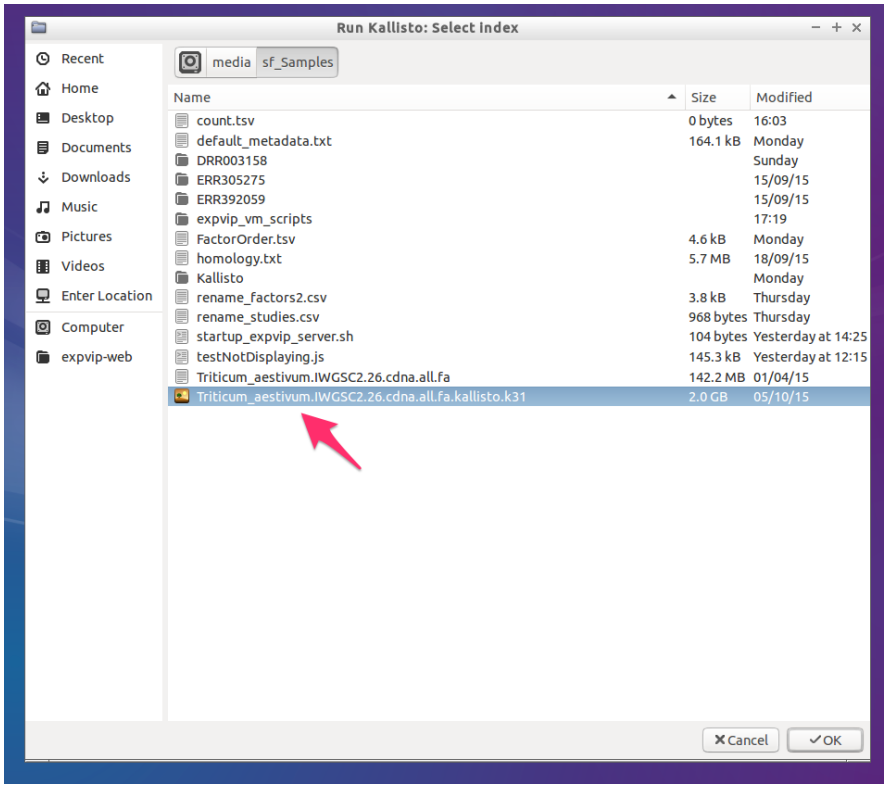
4. Get the name of the reference. This name must be the same used when loading the metadata



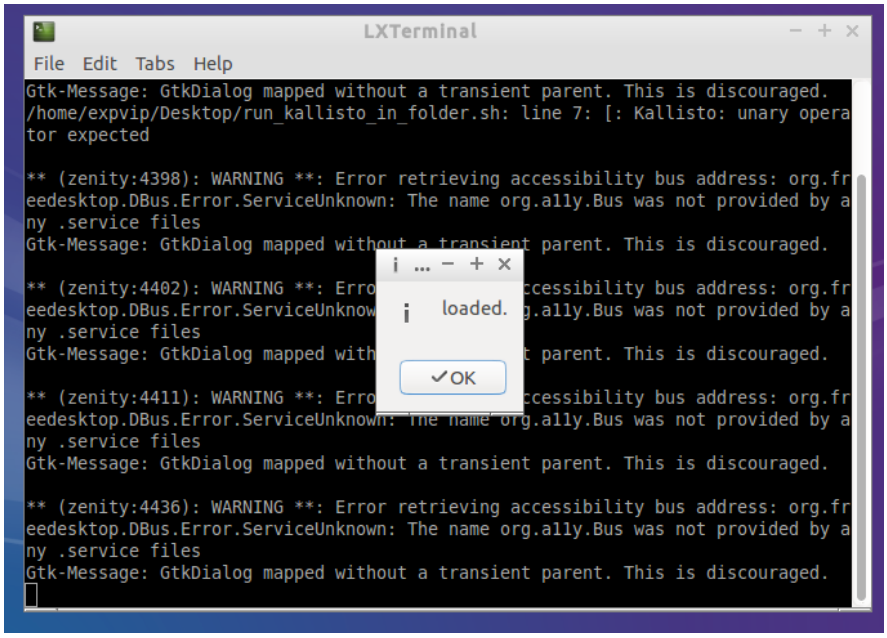
5. Select a folder with the folders containing the reads. The reads must be paired reads. The subfolder names must be the same as the `accession` used on the metadata. If a subfolder has an experiment that has been loaded already, it is not loaded.



6. Select the kallisto index



7. Wait for Kallisto to run and load the data



Repeat this with all the samples.

Rake task

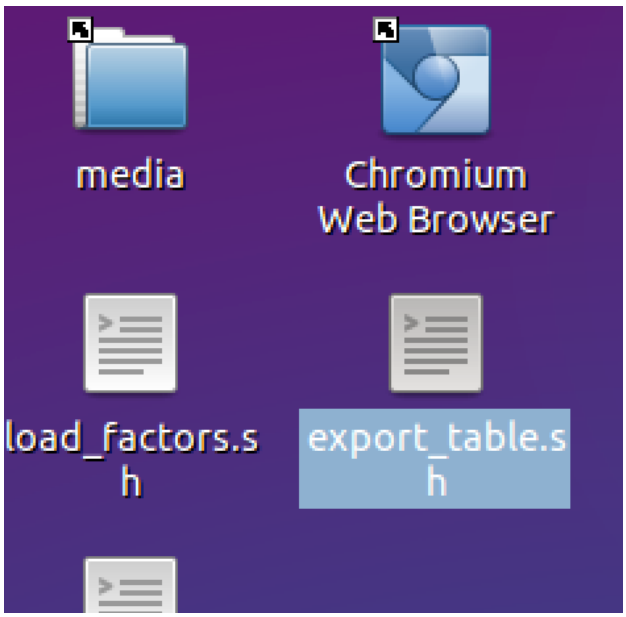
```
kallisto:runAndStorePairedFolder[kallistoIndex,input_folder,metaExperimentName,geneSetNam
```

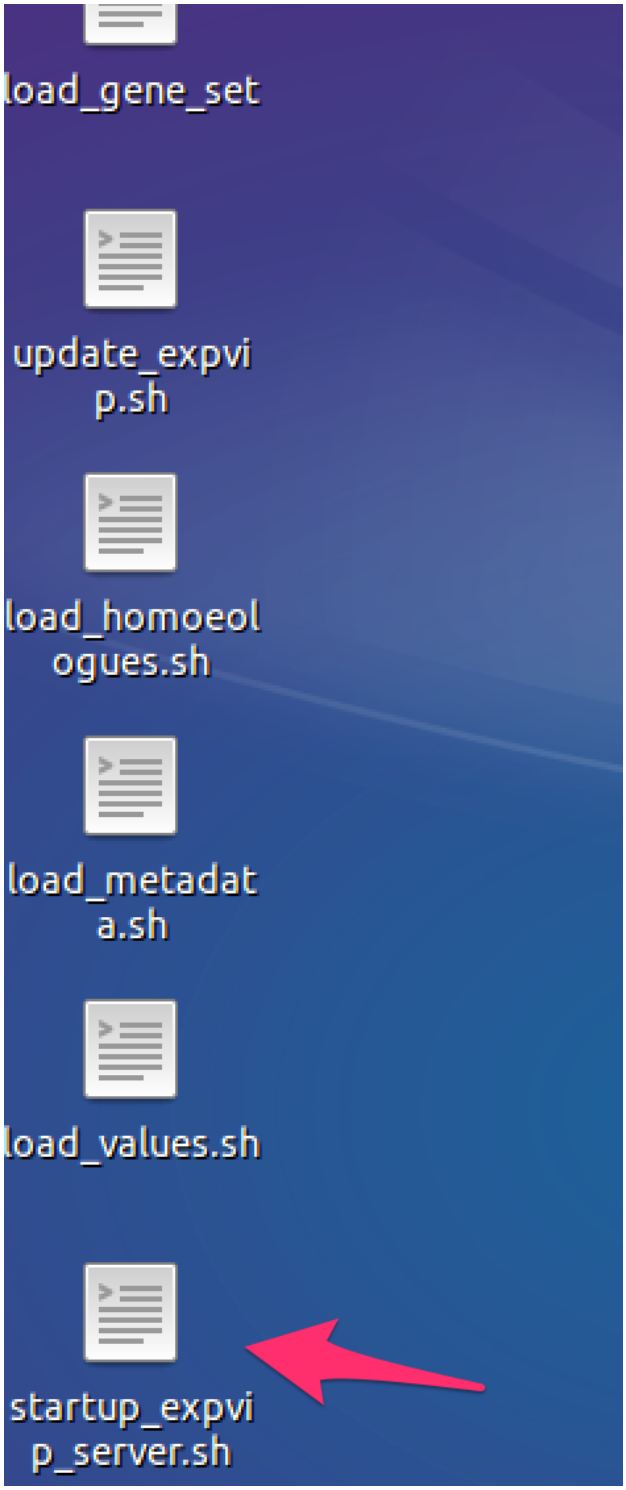
Where `metaExperimentName` is the name of the group of alignments under the same conditions and ` `geneSetName` is the name of the reference.

Starting expVIP web server

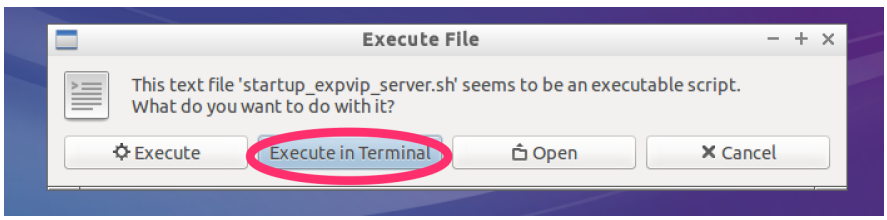
Once the data is loaded, you can visualize the the expression in the expVIP virtual machine.

1. Double click on `start_expvip_server.sh`

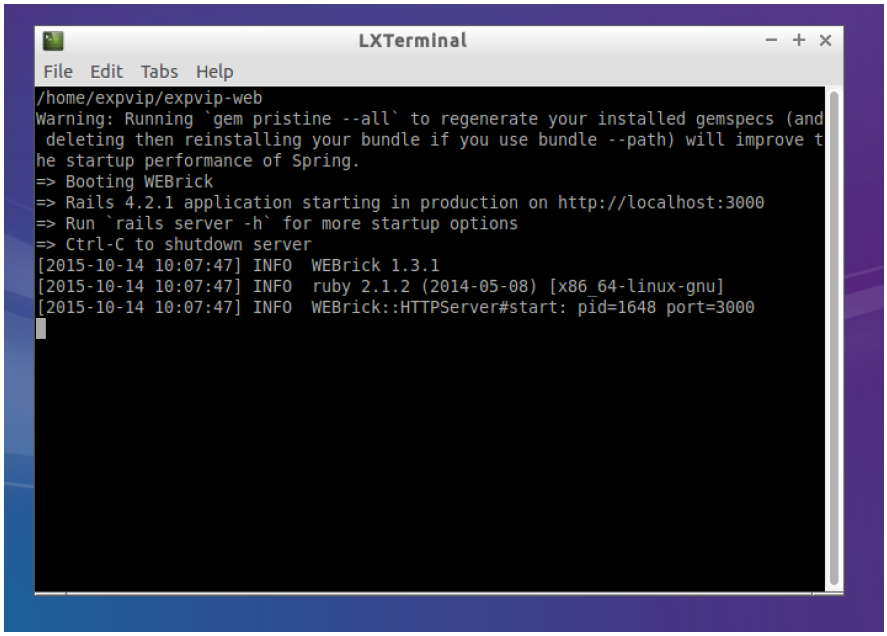




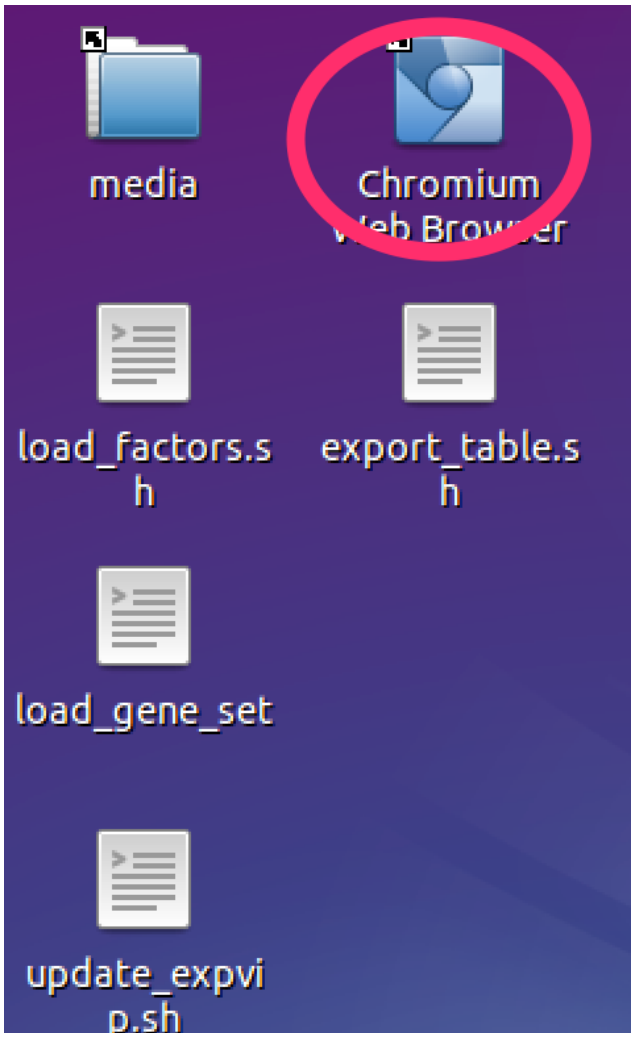
2. Click on `Execute on terminal`

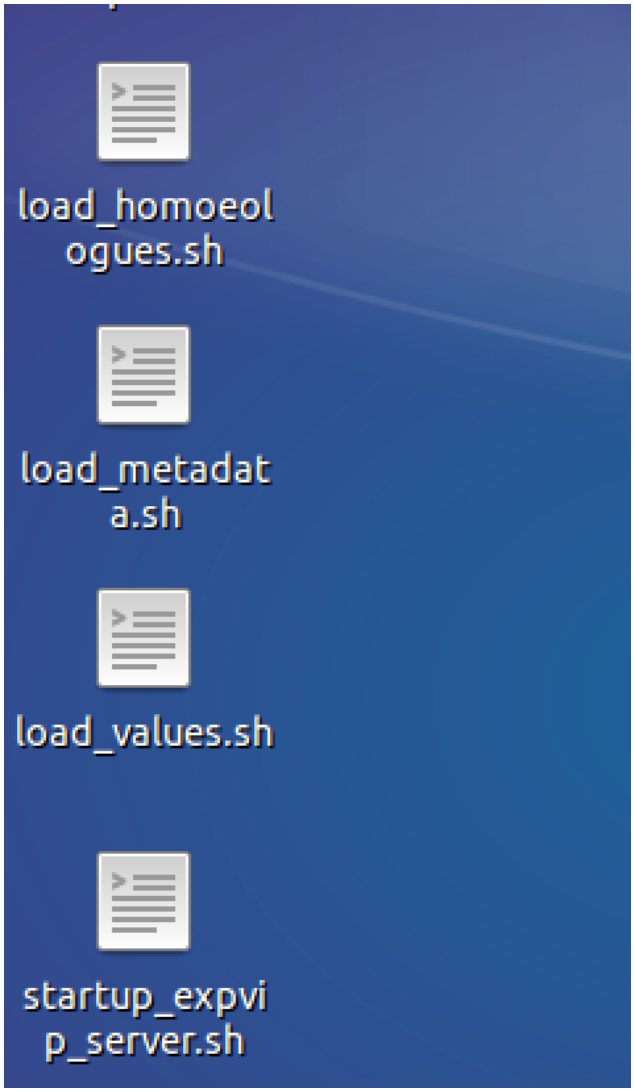


3. Wait fot the webserver to start. You know it is ready when the line
   `WEBrickHTTPServer#start: pid=xxxx port=3000` appers in the console

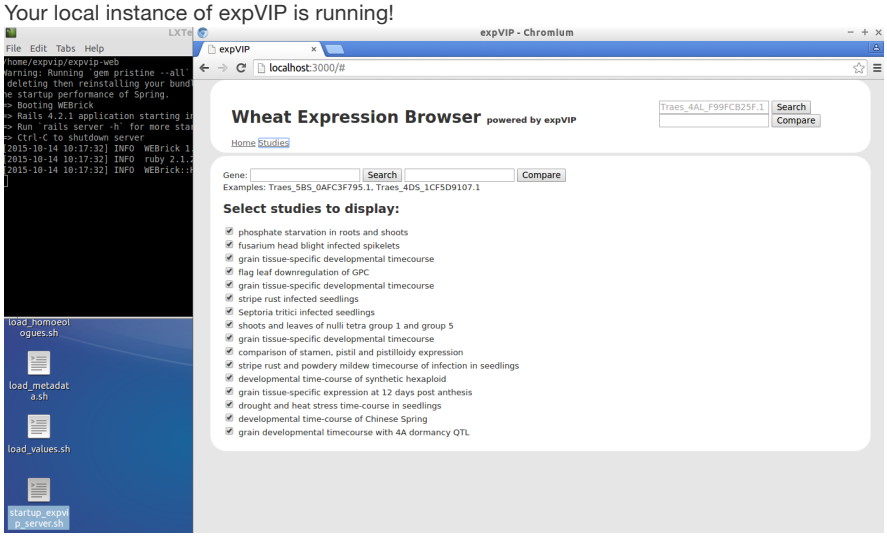


4. Double click in Chromium Web Broser.





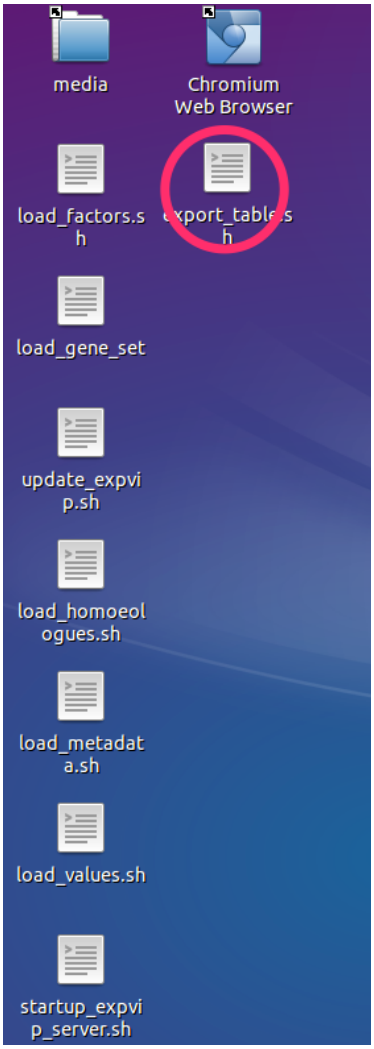
5. Your local instance of expVIP is running!



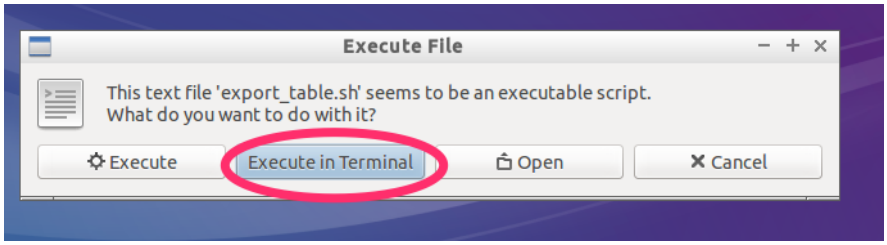
Export data

The data loaded in `expVIP` can be exported to be run in DESeq2, or any other software to do differential expression analysis.

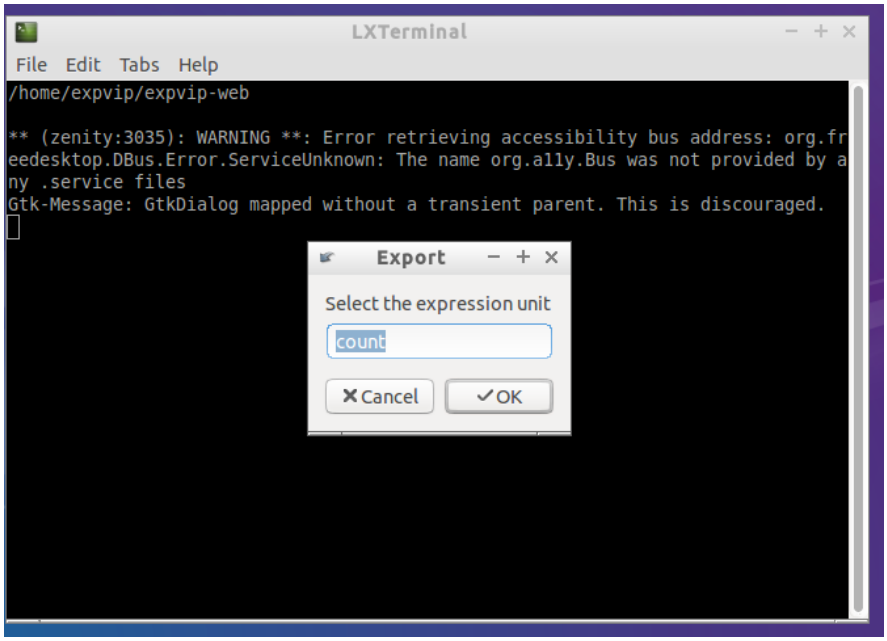
Wizard to export data



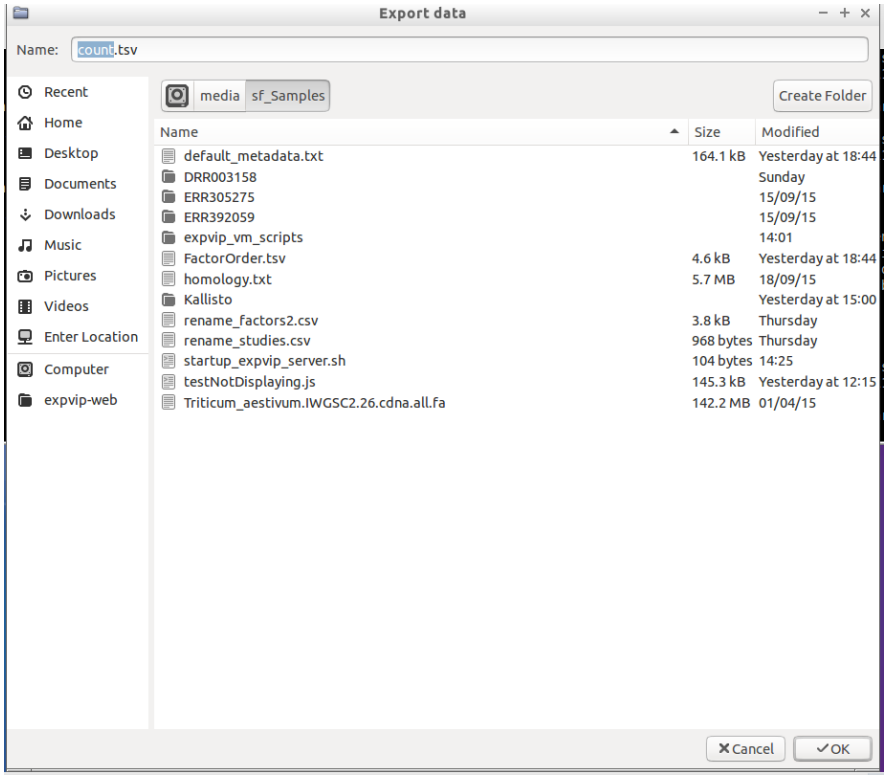
1. Double clicl on the the `export_tables.sh` script
2. Execute it on the terminal



3. Select the value you want to export. If you have used `Kallisto`, the available options are `count` and `tpm`. If you imported the data manually, it will be whatever units you inserted



4. Select a location for the output file. It is suggested to export it in the shared folder with the host machine.



Rake tast

```
rake "export:values[tpm,tpm.csv]"
```

Abundance files

To use Sleuth, the abundance files from the `Kallisto` runs can be grabed from the folder `kallisto` inside the folder with reads.

The abundance files for the runs in the VM and in wheat-expression.com can be found in thie here.

Troublshooting

If you get an error like this:

```
ActiveRecord::StatementInvalid: Mysql2::Error: Error writing file '/tmp/MYg0xdqm' (Errcod
```

You can try increasing the size of the virtual machine disk or install expVIP in a dedicated workstation. The best thing to do is to download the precalculated tables from the expVIP website and add the columns with your experiment at the end of the table.

This tutorial is based on the `Wheat Expression Browser` . However, the principles are the same for any transcriptome study which is powered by the expVIP graphical interface.

Home Page

The home page allows the user to insert a gene name to search and to define which studies are to be included in the visualisation interface. By default all studies are selected, but users can select/deselect a study by simply clicking on the specific button.

You can also compare expression between two genes by introducing both gene names in the boxes and pressing the `Compare` button.

Alternatively you can compare expression across multiple genes (up to 50) to generate a heatmap. You can add a list of genes separate by commas or one gene per line in the `Multiple genes` box.

All gene names are based on the transcriptome reference used for expVIP: for the case of the Wheat Expression Browser we used the IWGSC transcriptome available through Ensembl Plants release 26.

Visualisation interface

Single gene or two-gene comparison

Once the gene expression loads the page includes several features. These are shown below and explained point by point:

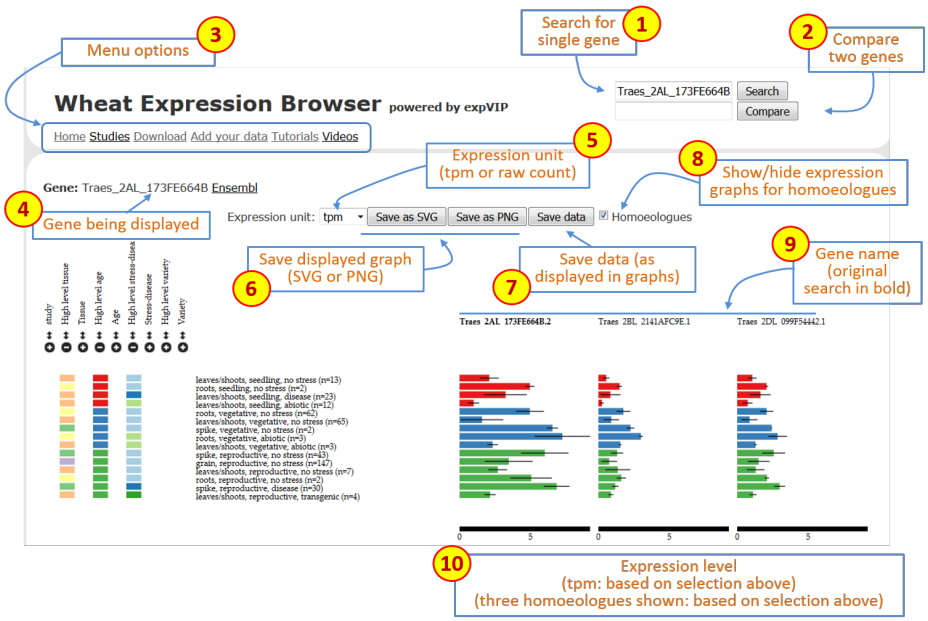


Figure 1: Overall description of features on Wheat Expression Browser

1. **`Search box`** : at any point you can type or copy a new gene name (based on Ensembl Plants nomenclature) and generate a new set of expression data.
2. **`Compare box`** : you can type a second gene name and press the `Compare` button to generate two expression graphs drawn at the same scale.
3. **`Menu options`** : this includes a series of links to different options:
    - **`Home`** : return to `home` screen.
    - **`Studies`** : opens up a popup screen with a summary and short description of each study and a link to manuscript.
    - **`Download`** : link to download all the wheat expression database including `tpm` and `counts` and associated metadata.
    - **`Add your data`** : link to GitHub to download virtual machine.
    - **`Tutorials`** : link to Wheat Expression Browser Tutorial.
    - **`Videos`** : link to Wheat Expression Browser Video Tutorial.
4. **`Gene`** : shows the gene which is currently being displayed with link to Ensembl Plants gene page.
5. **`Expression unit`** : allows user to select the expression unit used to visualise the expression data. This can be either “transcript per million (`tpm`)” or “estimated counts (`counts`)”. We have not provided RPKM given the inconsistencies generated across samples when using this measure. A detailed discussion can be found in Wagner *et al* (2012). It is important to mention that `tpm` is preferred over RPKM since it allows an easier comparison for abundances between samples. However it is important to stress that while `tpm` serves as a relative measure to compare genes across experiments, a proper normalisation and statistical analysis with differential gene expression programs must be performed. `expVIP` generates outputs which allow easy implementation of `sleuth` , `DESeq` and `EdgeR` .
6. **`Save graph`** : these two buttons allow users to save the current graphs in either `SVG` (to work on Adobe Illustrator) or as `PNG` files. The graphical file will render based on the current selection and order of factors as displayed on the screen.

7. `Save data` : this allows the user to download a `csv` file with the data based on the current selection and order of factors as displayed on the screen. The data will include the standard errors and the number of samples that make up each value.
8. `Homoeologues` : by clicking on this button, the `Wheat Expression Browser` will display the expression graphs of known homoeologues of the original primary gene. This gene name will remain in bold and the homoeologous graphs will be displayed according to A, B, D genome ordering. When homoeologues are displayed the same expression scale is used across graphs and the sorting and filtering of factors is simultaneous to allow easier comparison.
9. `Gene names` : gene name for corresponding graph. When homoeologues are shown the original gene used for the search is shown in bold.
10. `Expression level` : the expression level adjusts according to the expression of each set of gene homoeologues. The scale remains consistent across homoeologues to allow easier comparison. The values are based on the unit selected in the `expression unit` box (see point 5 above).

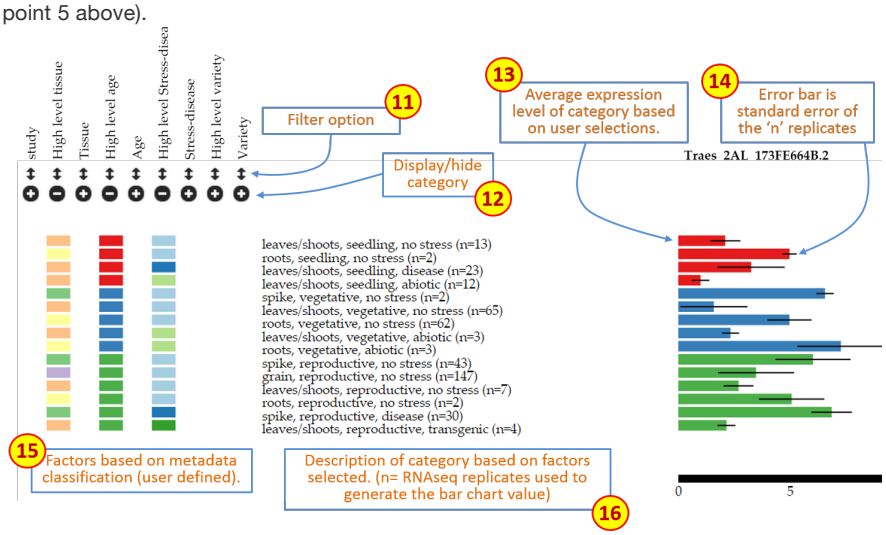


Figure 2: Overall description of features on Wheat Expression Browser (continued)

11. `Filter` : This feature open a pop-up window which reveals all the levels within the particular category. All levels are pre-selected, but users can choose to display specific levels by selecting or deselecting them accordingly. If a level is deselected, then the data associated with this factor is removed from the graph. Within the pop-up window levels can also be re-arranged according to the user's preference by dragging the level to the specific position within the pop-up window (see Features section below).
12. `Display/hide category` : Each individual category can be displayed or hidden by pressing the `+/-` button. When a category is displayed, the expression graphs will re-arrange according to the new category which has been introduced. If a category is hidden, then the graphs will also adjust accordingly. Data is not removed when doing this, rather it is grouped within the categories selected such that the total samples displayed remains the same. The colours within the category correspond to unique values or levels (up to 24 different colours) and are also used in the bar graphs corresponding to the expression data.
13. `Expression bars` : These bars represent the expression level of the "n" samples which are grouped according to the factors chosen based on the selection criteria (11 and 12 above). When hovering over the bar with the mouse a small tooltip will indicate the expression level (`tpm` or `counts`) and the standard error (sem) used for the error bars (see 14)
14. `Error bars` : Standard error of the means for the "n" expression values on which the bar graph is based.

15. `Factors` : Coloured rectangles represent the categories which are displayed according to the factors chosen based on the selection criteria (11 and 12 above). When hovering above the rectangles a tooltip will appear to show the long name of the level being examined.

16. `Description` : Text description of the factors chosen based on the selection criteria (11 and 12 above) and the number of RNAseq samples (n) which meet this specific criterion.

Multiple gene comparisons

17. `Expression unit` : For heatmaps, log2(tpm) is suggested as the expression unit as this provides better resolution to compare multiple genes across several categories.

18. `Heatmap` : Expression data is represented as a heatmap. As for single genes, categories can be sorted and filtered using the same tools. Gene names appear on the top of each column. Currently, up to 50 genes can be visualised in one heatmap. In Figure 3, for example, the two right-most genes are expressed solely in grains, with one being expressed to higher levels as suggested by the dark blue colour.

19. `Scale` : Colour scale for the expression values in the heatmap. The values adjust according to the highest tpm value being displayed within the current heatmap visualisation. Since tpm values below 2 are considered as very low expressed genes and log2 values of tpm<1 result in negative expression values, we forced tpm values below 1 to have a log2 value of cero (i.e. log2(<1)=0).

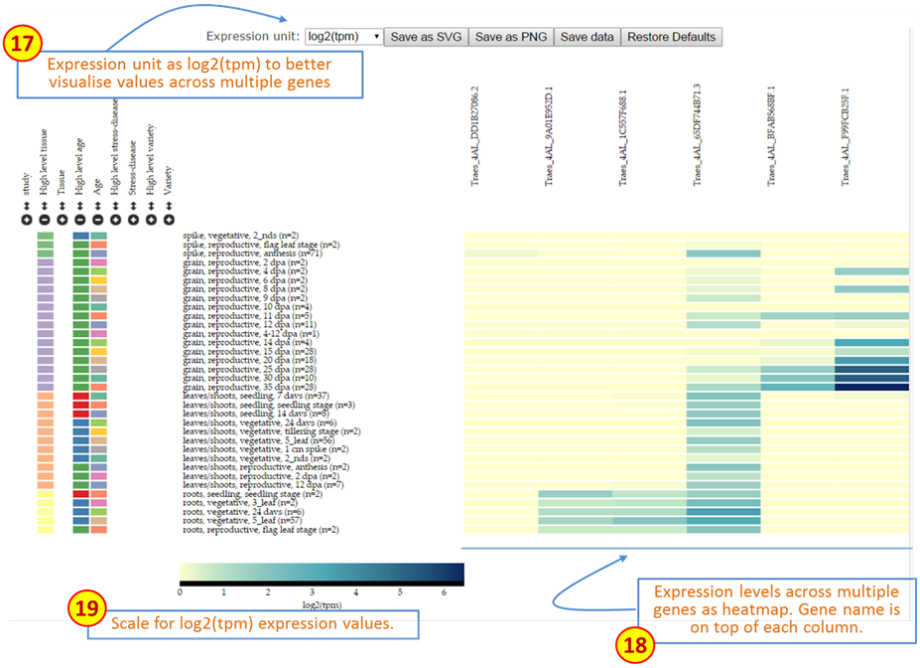


Figure 3: Description of features on Wheat Expression Browser using Multiple gene comparisons.

Features

Sorting

Factors can be sorted within each category in two ways.

1. The first is by simply clicking the mouse on top of the coloured rectangles underneath the

heading. For example in Figure 2 samples are sorted on `High level age` from `seedling` (red), `vegetative` (blue) to `reproductive` (green). If the user clicks on any of the coloured rectangles in the `High level tissue` category, then the graph is automatically reorganised based on this factor. In this case it includes four categories as defined by the user in the metadata and the bar graphs on the right hand side change colour according to the latest factor used for sorting. The previous factor used (in this case `high level age`) remains as a secondary sorting factor (Figure 3).

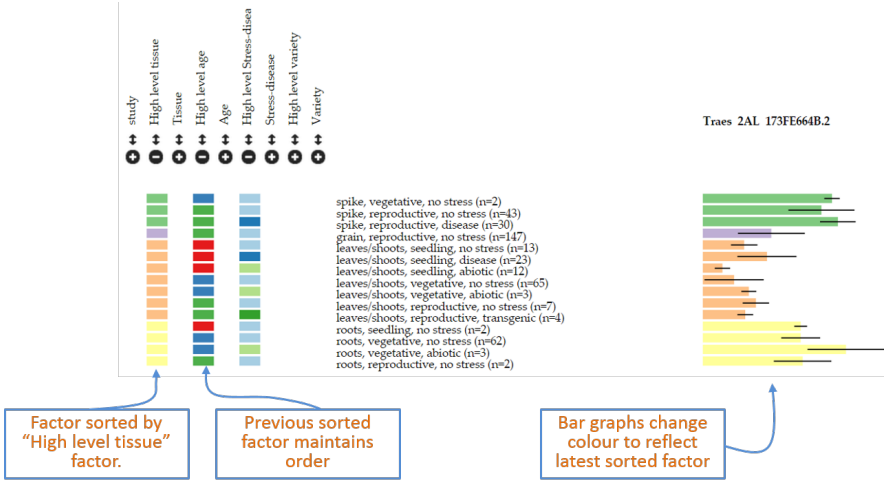


Figure 4: Example of new sorting of data based on clicking of rectangles within "high level tissue".

2. Alternatively, the user can define the exact order of factors within the browser interface. To do so the `filter` option (point 11 above) can be used. By clicking on the double arrow button the user opens a pop-up window which shows the levels within the factor. In this example by pressing the double-arrow underneath `high level tissue` a pop-up with four levels appears based on the order as determined in the user defined metadata (`spike`, `grain`, `leaves/shoots`, `roots`). To rearrange this, the user can simply click, hold and drag the level to the desired position. This will automatically re-arrange the data based on the new order and the corresponding graph and legends will follow suit. The bottom panel of Figure 5 shows a new order of `roots`, `leaves/shoots`, `spike` and `grain`.

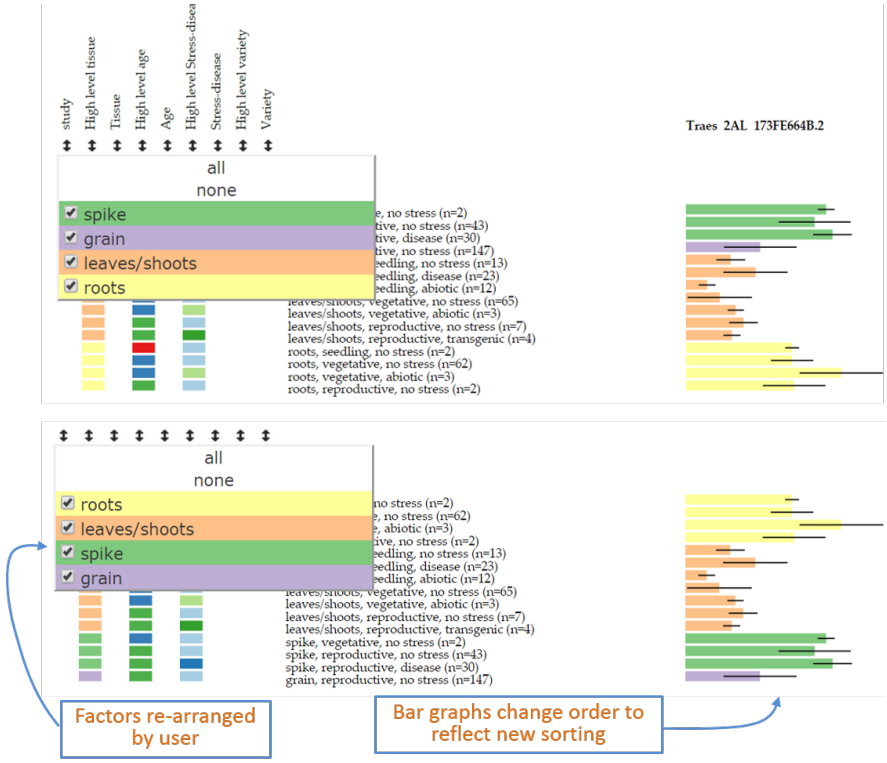


Figure 5: Example of sorting of data based on new user defined order within the filter pop-up window.

Filtering

In cases it may be required to remove certain samples from the visualisation. Note that displaying or hiding a category (point 12 above) does not remove the underlying data from the visualisation: this just simply groups the data within the selected category. Therefore to remove samples from the visualisation the user can open the filter pop-up as described for the `Sorting` option. Individual levels within the category can then be removed by using the "check-box" on the left hand side of the level name. By de-selecting a given level (in the example for Figure 6 we have deselected `leaves/shoots` and `spike`), samples defined as such will be removed from the analysis and will not be shown in the bar graphs. In Figure 6 now only two levels remain (`roots` and `grains`) and hence the bar graphs only show these two levels. Notice that the numbers of samples which comprise each bar graph are the same as those on Figure 5. The pop-up window also includes an `all` and `none` option to rapidly select/deselect individual samples. The filtering option can be used on any factor: for example to remove a complete study from the analysis the easiest way is to select the `study` filtering pop-up on the far left and deselect the study in question.

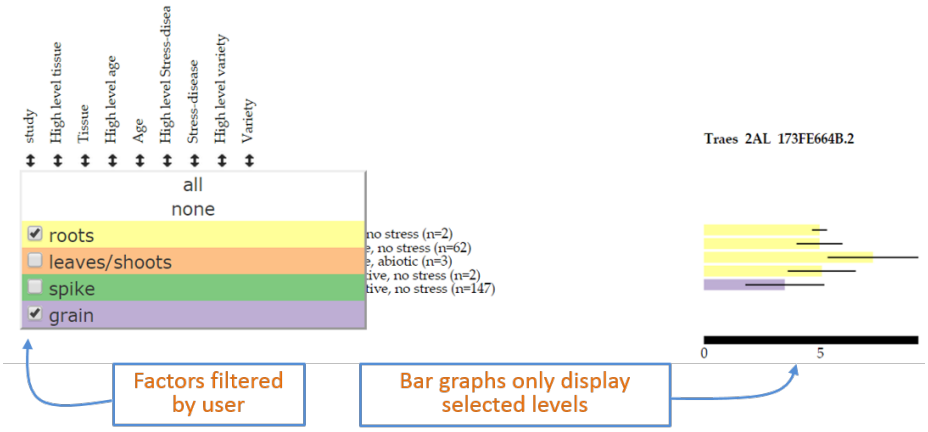


Figure 6: Example of filtering data based on user defined selection within the filter pop-up window.

Acronyms

ACID Atomicity, Consistency, Isolation and Durability. 114

AVS Avocet S. 10, 69, 71, 73–75, 77, 78, 85, 92, 99, 104

BC Back-cross. 16, 69, 75

BFR Bulk Frequency Ratio. 66, 77, 82, 96, 97, 104, 105

BSA Bulk Segregant Analysis. 65, 101, 103, 105

cDNA coding deoxyribonucleic acid. 67, 125

CS Chinese Spring. 24, 26, 153, 154

CSS Chinese Spring Chromosome arm survey sequence. 9, 26–28, 41, 53, 54, 60, 67, 75, 79, 82, 87, 89, 98, 99, 102, 103, 105, 151, 154, 160

CSV comma separated values. 49

DH Doubled Haploid. 16, 17, 28, 152

EBI European Bioinformatics Institute. 106, 140, 141

EI Earlham Institute. 146, 152

EST Encoding Sequence Tag. 26, 103

expVIP expression Visualisation and Integration Platform. 7, 8, 107, 116, 117, 119, 121, 122, 125, 130, 136, 138, 140, 141, 144–148, 158

FPKM Fragments Per Kilobase of transcript per Million of mapped reads. 117

GUI Graphical User Interface. 7, 131, 132, 134

HPC High Performance Computing. 143

indels insertions and deletions. 6, 38, 42, 44, 46

IUPAC International Union of Pure and Applied Chemistry. 9, 23, 73

IWGSC International Wheat Genome Sequencing Consortium. 24, 117, 141, 145, 147, 154

JIC John Innes Centre. 75, 77

MAS Marker Assisted Selection. 90

MVC Model View Controller. 7, 114–116, 130

NCBI National Center for Biotechnology Information. 26, 60

NGS Next Generation Sequencing. 19, 23, 27, 66, 79, 102, 105

NIL Near Isogenic Line. 16, 64, 69, 75, 103

PCR Polymerase Chain Reaction. 32, 33, 60

PST *Puccinia striiformis* f. sp. *tritici*. 57, 64

QTL Quantitative Trait Locus. 79

RDBMS Relational Database Management System. 114, 115, 121

RIL Recombinant Inbred Line. 16

RoR Ruby on Rails. 115, 116, 125, 128, 130

RPKM Reads Per Kilobase of transcript per Million of mapped reads. 24, 117, 141, 142

SNP Single Nucleotide Polymorphism. 23, 27, 28, 32, 39, 62, 67, 73–75, 77, 82, 87, 104, 105, 152, 159

SQL Standard Query Language. 112, 114

SSR Simple Sequence Repeat. 57, 90

T-DBG transcriptome de Bruijn Graph. 110, 144

TPM Transcripts per Million of mapped reads. 24, 117, 127, 132, 137, 142

UCW University of California Wheat. 26, 67, 73–75

WGS whole genome shotgun. 24, 26

Yr15 Avocet + *Yr15*. 7, 10, 69–71, 73–75, 77–79, 82, 83, 85, 89, 90, 92, 93, 99, 102, 151

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