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Chapter 1

Introduction

In order to produce bioinformatic software that is powerful and usable it is required an understanding of both: the biological processes to solve and; the computational methods and software development practices.

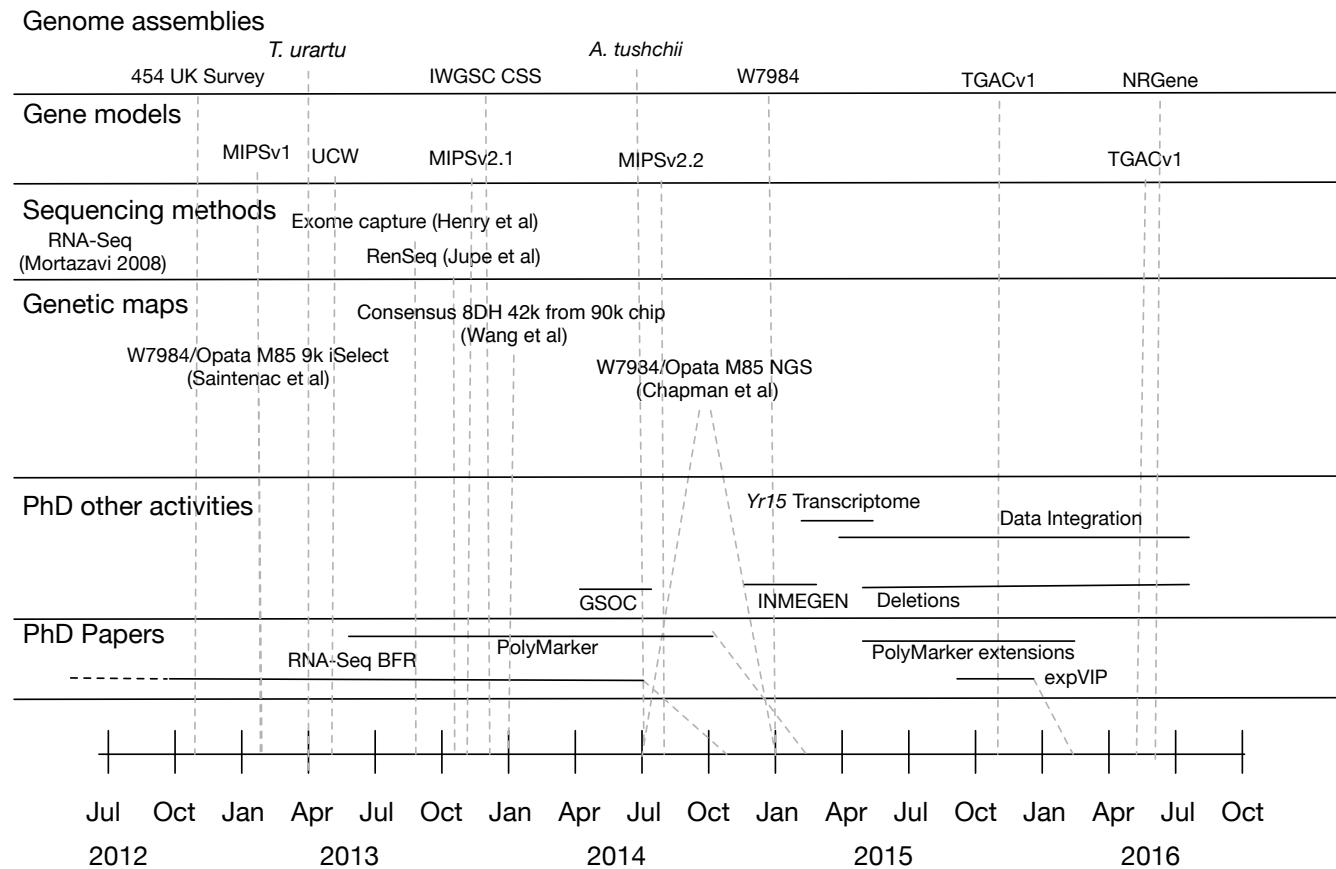


Figure 1.1: Timeline of the projects carried on during this PhD and the wheat resources that were released in the same period of time.

1.1 Wheat Breeding

An overview of how breeding is carried on currently, the different sources of genetic diversity and the relevance of fixing agriculturally important traits.

1.2 Wheat Genetics

The section describes alleles an the concept of gene, both as a locus in the genome (Quantitative Trait Locus, QTL) and an specific transcript (central dogma of molecular biology). Finally, it discusses traditional Mendelian inheritance and the effect of polyploidy. Some of this is described in the Yr15 chapter, maybe it is not needed any more here.

1.3 Polyploidy and Wheat

A polyploid species contains more than one set of related genomes, that may come from a chromosomal duplication or from an hybridization with a related species. *Triticum aestivum* (bread wheat) has gone trough an specialisation event and two major hybridization events. Initially, an unknown species first evolved in two different species around 7 million years ago to form the A and B genomes, whose closest known relative are *Triticum urartu* and *Aegelopolis speltoides*. As both ancestral wheat were able to cross, at some point around 5.5 million years ago the D genome arose, *Aegelopolis tauschii*. Then, less than 800 thousand years ago the ancient species carrying the A and B genomes hybridized and formed a tetraploid wheat, *Triticum turgidum* (pasta wheat). A final event occurred less than 400 thousand years ago, when pasta wheat hybridized with the carrier of the D genome, leading to bread wheat (Figure 1.3, Marcussen et al. 2014).

Because bread wheat contains three independent copies of its genome, the expectation is to have three homoeologues for each gene.

Talk
about
par-
alogues.

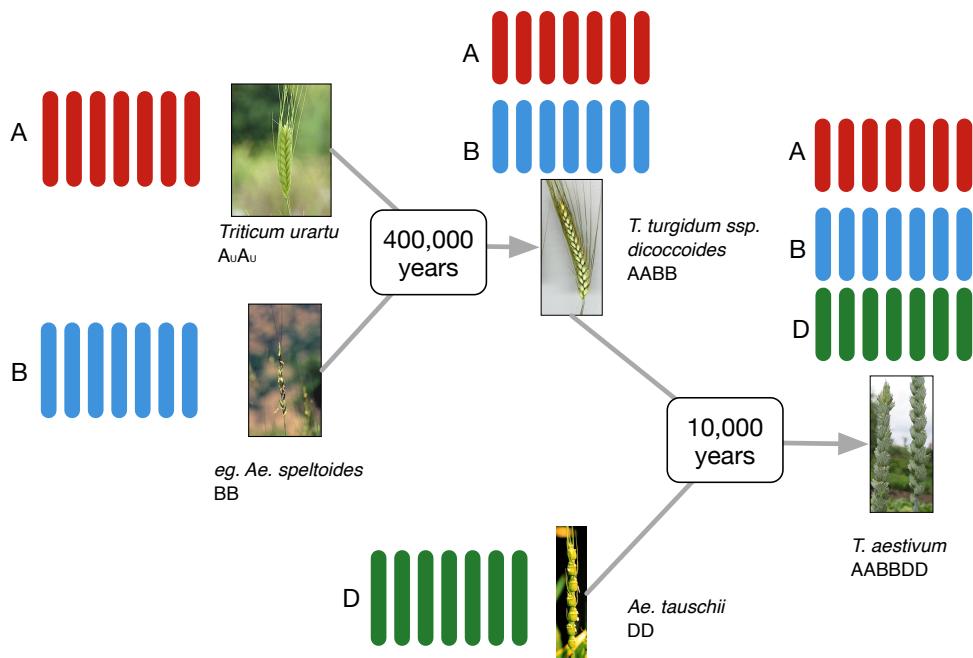


Figure 1.2: Hybridizations that lead to bread wheat *T. aestivum*.

1.4 Wheat Genomics

A description of the current status of the wheat genome (Mayer et al. (2014), Chapman et al. (2015)), the different available assemblies and approaches to sort the scaffolds (Genome Zipper, the various genetic maps).

1.5 Sequencing

The Human Genome Project used Sanger sequencing Lander et al. (2001). This technology is the current gold standard in terms of quality of the sequence. It evolved from electrophoresis gels where the bands represented bases to a fully automated technique. However, the throughput is limited and doing genome wide analysis has prohibitive costs. In the second half of the 2000s high-throughput sequencing technologies emerged which had reduced the cost of sequencing. The main principle of the second-generation sequencing is to produce clusters of clones (i.e. ePCR), fix them in a plate and then add bases with a fluorescent marker. The reaction happens in parallel in millions of clusters at the same time. With each cycle, a picture is taken, showing the fluorescence of each base.

Then, image processing algorithms find where in the image the clusters are and the bases are called. At this scale, the volume and complexity of the information is not trivial to manipulate, hence computing is required.

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 followingMyllykangas et al. (2012); Metzker (2010); Shendure and Ji (2008); Hutchison (2007):

Single end When the fragments are short, it is possible to just sequence from the 5'-end the read.

Read Pairs When the sample consists fragments of up to 500bp, it is possible to read the 5' end up to the read length were the quality starts to drop, the molecule can be turned upside down, reverse complemented and sequence backwards. It is not required, but ideally, the fragments sequenced with read pairs should be selected to have an homogenous size. The reads are in opposite orientation relative to each other.

Overlapping Read Pairs are a variation to read pairs, where the size of the fragment is shorter than two times the read length. This allow an alignment between the two fragments to get an longer read with the limitations of the instrument.

Mate pairs are 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.

There are several types of experiments that can be analysed with hight throughput sequencing, accordingly, different protocols for the sample preparation exist. The following is a short list of some of them

Whole genome shotgun When a sample is prepared for WGS, the DNA is extracted and chopped in fragments and sequenced. The reads obtained are, in principle, randomly distributed across the whole genome

RNA-Seq . Instead of sequencing DNA, mRNA is captured and sequenced. The fragments are not amplified in any way, to enable a portrait of the gene expression levels.

ChIP-SEQ . Chromatin Immunoprecipitation is used to find relationships between proteins and DNA sequence. It is useful to find transcription factors and replication-related proteins.

Amplicon sequencing . Used primarily to do barcoding of species. A known gene is amplified (i.e. 16S) with the intention of characterising the species present in the sample.

Metagenomic capture From a mixed sample (soil, root, animal fluids) all the DNA is extracted and sequenced, this gives a snapshot of the microbial community in the sample

RAD-seq Restriction site associated DNA markers are useful to do population analysis. The technique focus on sequencing regions around restriction sites and the variations around them can be used to genotype individuals.

Exon capture The DNA is extracted and baits are used to attract the regions with motif common around exons. This allows to sequence only the genes and regions near them.

The different sequencing technologies available as of 2013 have different yields, advantages and disadvantages, as described below:

Illumina Each fragment is amplified using bridge amplification over and over in the same place in the plate to form clusters. After the clusters are formed, a last cycle of amplification is carried on with the bases being added to the template, with the intervention of a polymerase, have a fluorescent marker which make the cluster glow depending on the added base. It adds one base per cycle. With a read length between 75bp and 250bp is currently the most widely adopted platform. As a de facto standard, many tools exist to cope bioinformatically with the biases of the machine. The run takes 4 or 9 days, depending on days, depending if one or two reads are generated for each fragment. It produces up to 35 gigabases per run.

SOLiD The preparation of the fragments is similar to Illumina, however, when adding the bases they are added in pairs. This technique is called sequencing by ligation as it uses a DNA Ligase, as opposed to a polymerase, to determine the transition between bases. The resulting sequence is not in base space, but in colour space, which represents the transition state between bases. This technique is robust for finding SNPs when you have a good reference where to align the reads. However, the number of tools available and the research done to analyse sequences in colour space is low compared to the tools using base space. The runs take between one and two weeks to complete, with a yield of up to 50 gigabases per run. The read length can be up to 50 bases.

Roche/454 The fragments are cloned in beads, which then fall in wells in the slide. The sequencing is done by adding nucleotides in a determined order. The next nucleotides to be added in the reaction contain a fluorescent marker. The bases are not added one by one, but all the bases that are the same are added together. The amount of glow on each well can tell how many times a base is added. As the glow is not a discrete number, when a long homopolymer appears (above 5 bases) the likelihood of having a wrong count of the homopolymer is increased. The average read length varies between 300 and 700bp. A run usually takes half a day, but it only yields 0.45 gigabases. The cost of the reagents is relatively expensive, but if the experiment requires longer reads it is a good option.

PacBio Opposed to all the previous technologies, Pacific Biosciences has developed a sequencing technology where the molecules doesn't need to be PCR amplified before the sequencing. The glass slide used contains wells with a depth of 100nm where a polymerase lays at the bottom. The nucleotides to be added have a fluorescent marker that is freed when the polymerase adds the nucleotide, releasing a light signal, which then can be captured from the bottom of the glass. The error rate for this technology is still high (about 10% of the bases are miscalled), however reading several times the same molecule reduce the error rate. The main advantage is that the reads can be over 1kbp.

OpGen Additionally, high-throughput optical mapping technologies, like OpGen, are becoming accessible. The maps are done by fixing single molecules of DNA are held on a slide. Then, restriction enzymes targeted to specific digestion sites cut the fragment and fluorescent markers are added to the ends of the fragments. Finally, the fragments are visualised and the size of the molecules is measured by the distance between fluorescent points in the slide. This is done with several fragments at the same time. Then, the distances between restriction sizes can be compared across all the fragments to generate a consensus. Finally, if you have contigs from other technologies, it is possible to complement the information and get better assemblies. Even without the contigs, the data can be used to compare translocations within strains of different bacteria or homologous species at a chromosome level.

ION Torrent (Do some research on newer sequencing things)

1.6 Sequence analysis

This section discusses the criteria to decide analysis done after sequencing, when to do re-alignments or *de novo* assemblies, how to do SNP calling in diploid and polyploid organisms and the bulk frequency ratios.

DNA sequence alone is not alone to enough to understand the biology behind, a context is required. There are databases like Ensembl and NCBI that act as repositories of the known public sequences.

From the computational point of view, the problem can be viewed as a string matching. The Smith-WatermanSmith and Waterman (1981) and Needleman-WunschNeedleman and Wunsch (1970) algorithms are the gold standard in terms 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 mn where m is the target sequence and n is the query sequence. To scale this to a manageable problem algorithms like BLAST index the references and use heuristics to make the search more manageable, with some penalty in the accuracy. This

alignments tools are useful for long stretches of DNA (like cDNA or contigs) Altschul et al. (1990).

TODO: List of global aligners -BLAST -BLAT -Exonerate -nucmer
-MAFFT -Clustal

When looking at a protein level, where the sequences may be only loosely similar, Hidden Markov Models (HMM) are used to search for protein families. This can be useful to annotate putative proteins and their functions. HMMs require a training dataset, where proteins are previously annotated and the reference is a model encoding the characteristics of a family, with associated probabilities. Hence, this technique is something between a sequences aligner and a classifier Eddy (2004).

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 in advantage 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 you have your reads aligned to a reference you can do more analysis, depending 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 modularised 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).

1.6.1 Ambiguity Codes

Make a table with the ambiguity codes and why they are useful.

1.6.2 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 high-throughput sequencing is RNA-Seq. This technique captures the messenger RNA in the tissue being studied and sequenced. The premise is that you will find a gene more expressed if it is being used by the organism. Some proteins with a vital role for the cell are always expressed (i.e. RuBisCO for carbon fixation in

plantsGM (2000)). On the simplest of the experiments you would need two datasets to compare, one with the gene being looked expressed and one where it is not. The expression can come from different environmental conditions, development stage or different genotypes.Mortazavi et al. (2008)

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 per Million mapped reads (RPKM) or the Transcripts per Million (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.

1.7 Wheat specific resources resources

Gene models -UniGene -UCW Gene models -Gene annotation IWGSC
-Gene annotation TGACv1

Genetic maps -Wang -Chapman/PopSeq (is the same population, improved)

Markers -90k -820k -MASwheat/SRR

Portal -CeralsDB -MASWheat -Ensembl -Wheat-expression

Assemblies -Chapman -IWGSC -TGACv1 -NRGene (unpublished?)
-454 Liverpool

A compilation of the currently available resource for wheat genetics and genomics. MAS wheat, CerealsDB, Ensembl, etc.

1.8 Computer Programming

Why Ruby and javascript? -Ruby -BioRuby -JavaScript -BioJS -Rails.
-SQL -D3
-lambda functions -functions/methods

1.8.1 Computer science used for this thesis

The following subsections give a brief overview of computer science concepts used in the thesis for the development of PolyMarker (Chapter 2), expVIP (Chapter 4) and, the algorithms for the data analysis for the marker development for *Yr15* (Chapter 3).

Functions

Hash tables

Object Orientated

Web development vs desktop development

-Containers, as in AWT.

Chapter 2

PolyMarker: A fast polyploid primer design pipeline

In modern breeding programs SNP markers are a prevalent technology to select seeds containing a particular locus linked to a trait (ie. a marker linked to a resistance gene, see Chapter 3). SNP marker are an specific case of Polymerase Chain Reaction (PCR) amplification with two competing sequences from different alleles are amplified.

In general, PCR amplification is a technique that can be used to copy several times a fragment of DNA. 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 when the DNA molecule is melted in individual strands with an increase of temperature. Then, the temperature is dropped so the primers anneal to the DNA strands. 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).

A technology used for SNP markers is KASP, the original target technology for PolyMarker. The assays consists on triplets of primers, having a primer for each allele and a common primer that will amplify regardless of the allele. The allelic primers have at the 5'-end a tail, HEX



Figure 2.1: PCR Diagram. PCR is used to amplify a region of the DNA (green bar). To 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.

(5' GAAGGTCGGAGTCAACGGATT 3') or FAM (5' GAAGGTGAC-CAAGTTCATGCT 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. On KASP assays, the distance between the left and right primers is 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 failed, because poor amplification or because all the samples have the same genotype, there are no distinguishable clusters (Figure 2.2c; LGC Genomics 2014).

One of the main challenges of working with polyploid species is the design of genome specific molecular markers. On hexaploid wheat, most of the genes have at three homoeologues copies, one for each genome (See section 1.3). The similarity between homoeologues is around 98%, which represent around 1 mismatch for every 50 bp. This means that a primer in a conserved region of 21 bases target any of the homoeologues if it doesn't 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 has a base that is unique to the D genome, hence the product is genome specific.

A variation between homoeologues in the primers is not enough to guarantee that the amplification is going to be genome specific. The

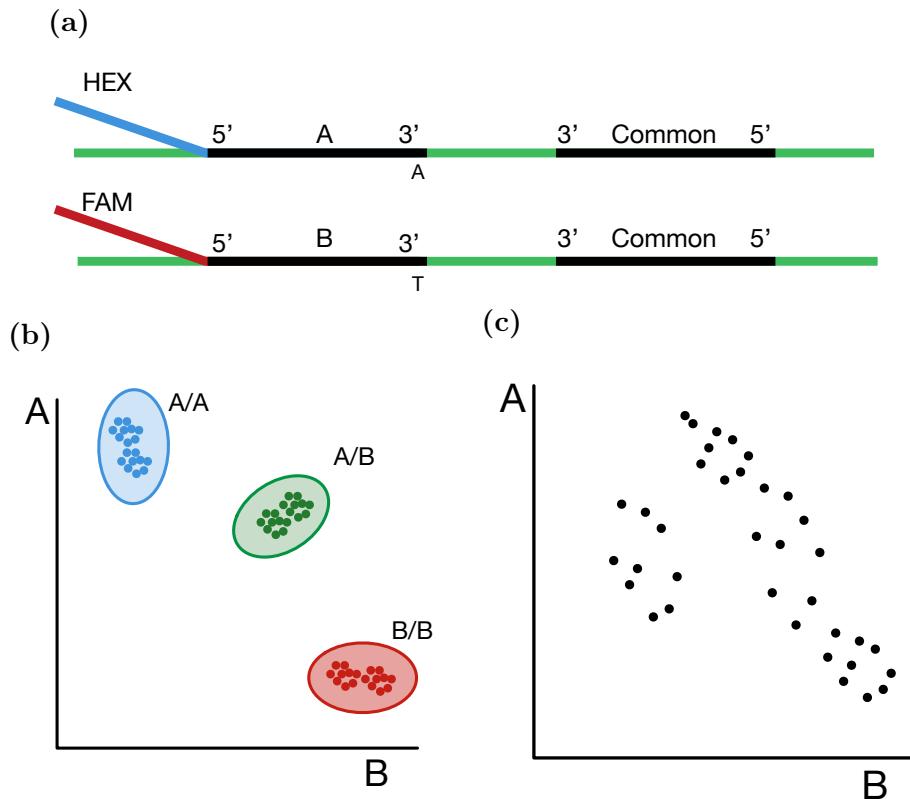


Figure 2.2: Kasp Assays (a) A KASP assay consists on three primers. Primers A and B are specific for certain allele and the HEX and FAM tails are added at the 5'-end on each primer. The common primer amplifies both possible products. The SNP is an A/T, the only difference between alleles. (b) Ideal KASP results of samples containing only homozygous samples. 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 of a failed experiment were clear clusters between samples are missing.

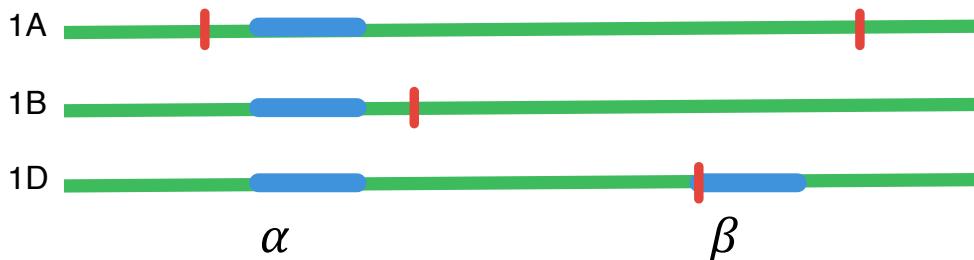


Figure 2.3: Target of genome specific primer. Primers selected randomly (blue lines) can bind to any of the three homoeologous regions if they fall on regions without variations between them (red vertical lines). The α primer doesn't contain any variation between chromosomes, hence it will bind to the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

```

Chromosome 1A  cgcatttgcgcgcgataccggcgctGtggaatatttgcagcgaaggcgtg
Chromosome 1B  cgcatttacgcgcgcgataccggcgctTtggaatatttgc---gaaggcgtg
Chromosoom 1D  c--atttgcgcTgcgataccggcgctGtggaatatttgcagcgaaggcgtg

cgataccggcgctTtgg  Mismatch at 3' end = Strong specificity
cgataccggcgctTtgg  Mismatch at 2nd position = OK specificity
cgataccggcgctTtgg  Mismatch at 3rd position = specificity not too strong
cgataccggcgctTtgg  Mismatch at 4th position = does not provide specificity

```

Figure 2.4: Effect of position of variation on primer specificity. Several candidates to design a genome specific primer for chromosome 1B. 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.

polymerase is more sensible 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; OK, when the variation is on the 2nd position; not too strong 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 constraints needed to produce 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 these tools take a reference sequence and make some sort of index 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 1.6.2). The results are aligned to the target and may include sequence only from one exon, but not the adjacent intron, hence it is necessary to make a local alignment.
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 to the target reference and using a program like `mafft` (Katoh and

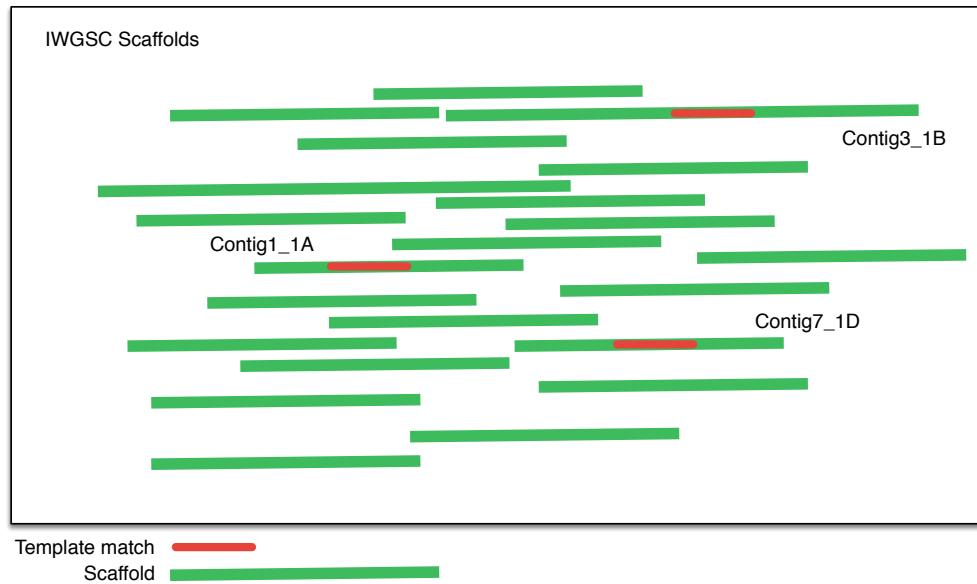


Figure 2.5: Global search of templates in the reference contigs.

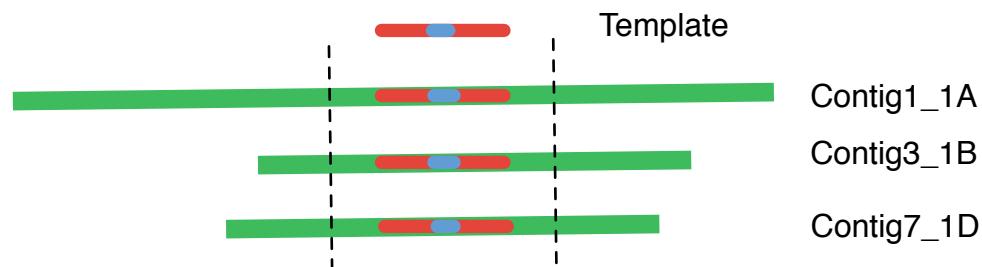


Figure 2.6: Selected regions around the SNP on every chromosome. The blue line represents the position of the SNP.

Standley, 2013) or **clustal** (Higgins and Sharp, 1988). These tools are based on aligning all the possible sequences in pairs all the possible pairs. 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 physiochemical properties that ensure the amplification. The melting temperature needs to be in the range where the DNA will separate, but not too high that reaching 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).

Since 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, I hypothesize that it is possible to automate the process. On that premise, I developed PolyMarker, a pipeline that takes the reference genome and a list of SNPs and produces genome-specific primers (Ramirez-Gonzalez et al., 2015a).

If possible,
expand
and add
diagram

2.1 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 1.7). 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 find the best primer pair that contains 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-polyplloid-tools**, the source code is available in: <https://github.com/TGAC/bioruby-polyplloid-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. 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

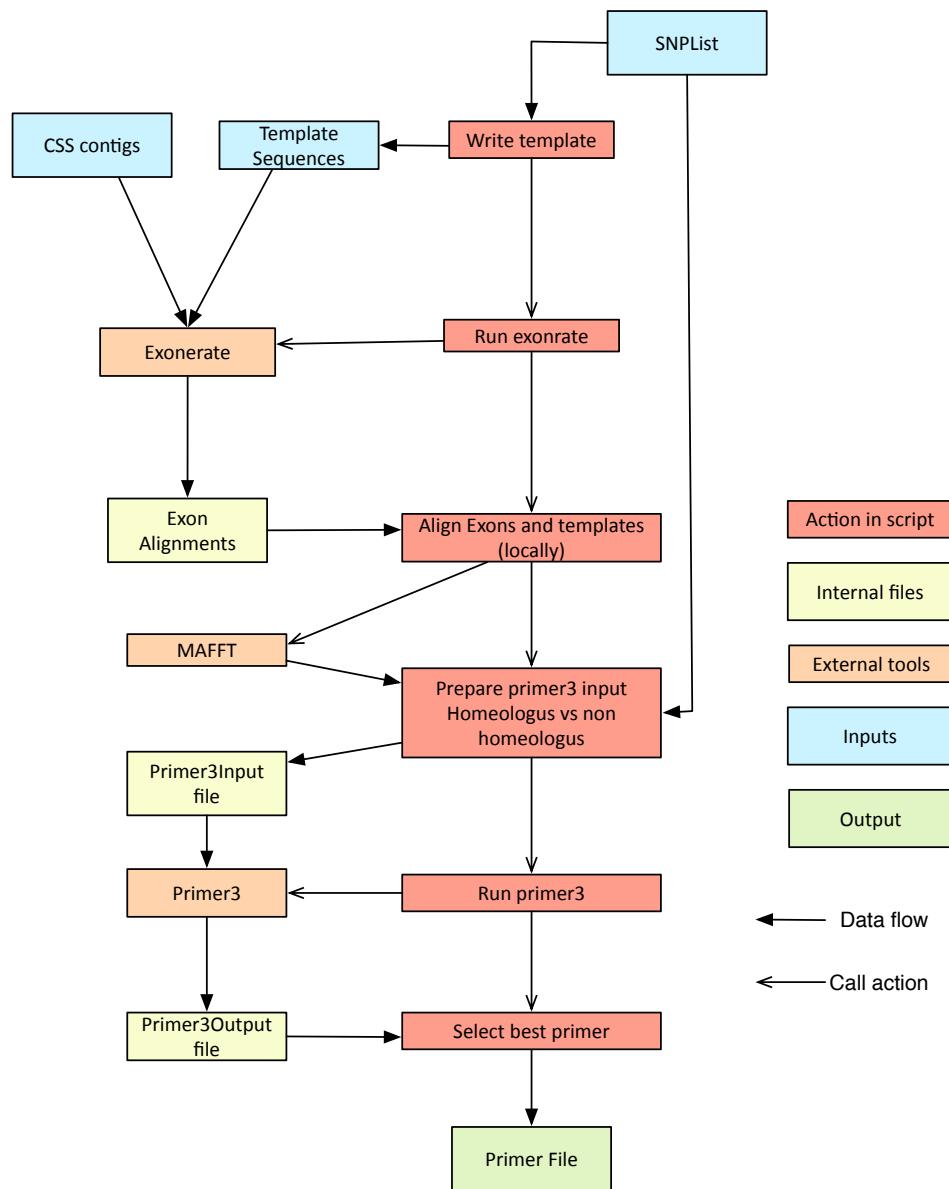


Figure 2.7: Steps and tools called by PolyMarker. The colour of the boxes represent: the step is an action inside the script(red); actions of the script(light red); temporary files(yellow); inputs(blue) and; outputs(green)

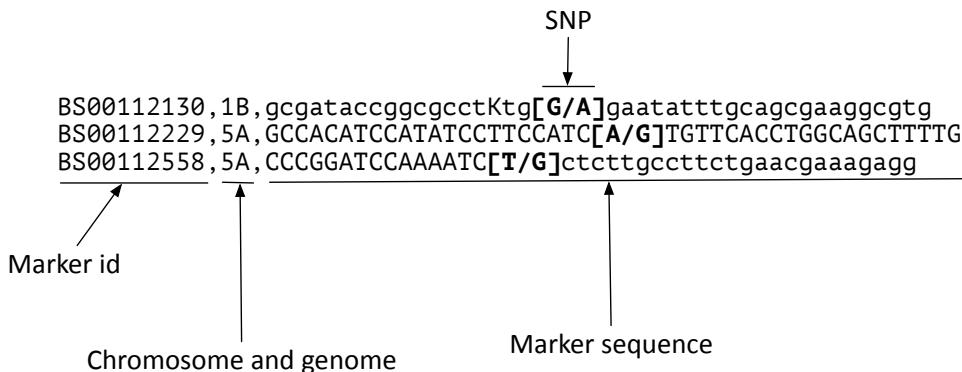


Figure 2.8: PolyMarker input. The alternative alleles are surrounded by brackets. The rest of the figures are based on BS00112130, renamed as SNP-1.

regions that diverge near the SNP, as when the variation is near an intron-exon junction.

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, one 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 follows: \%S the minimum 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.

```

SNP-1 A    cgcatttGcgcgYgcgataccggcgctKtgGaatatttcagcgaaggcgtg
SNP-1 B    cgcatttAcgcgYgcgataccggcgctKtgAaatatttcagcgaaggcgtg
IWGSC-1A   cgcatttgcgcgcgataccggcgctgtggaaatatttcagcgaaggcgtg
IWGSC-1B   cgcatttacgcgcgcgataccggcgctttggaaatatttcagcgaaggcgtg
IWGSC-1D   catttgcgcgTgcgataccggcgctgtggaaatatttcagcgaaggcgtg

```

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

The flanking sequence may contain indels and the sequences don't align naturally (Figure 2.9). The following parameters can be adjusted to extend the functionality of PolyMarker: Minimum Identity to designs for organisms with homoeologus 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 `exonrate` won't pay attention to the intron-exon junctions).

Each SNP marker is represented on the Bio::PolypliodTools::SNP class, containing the flakning sequence, the position of the SNP, multiple alignments and primers. For each step step there is a container (See container definition in Section 1.8.1) that holds the SNP set and parses each output for all the called programs. The container for exonrate is `BIO:PolypliodTools::ExonContainer`. The hits with the SNP is 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 `exonrate` output and add it to the corresponding SNP (Listing 2.1).

Maybe explain the architecture before getting to the pipeline, or after

```
1 def add_alignments(opts=Hash.new)
2   opts = { :min_identity=>90 }.merge!(opts)
3   exonerate_filename = opts[:exonerate_file]
4   File.open(exonerate_filename) do |f|
5     f.each_line do |line|
6       record=Bio::DB::Exonerate::Alignment.parse_custom(
7         line)
8       if record and record.identity>=opts[:min_identity]
9         snp_array = @snp_map[record.query_id]
10        snp_array.each do |snp|
11          if snp.position.between?( (record.query_start + 1),
12            record.query_end)
13            exon=record.exon_on_gene_position(snp.position)
14            snp.add_exon(exon, arm_selection.call(record,
15              target_id))
16          end
17        end
18      end
19    end
20  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 sequence (Listing 2.2).

```
1 def add_exon(exon, arm)
2   @exon_list[arm] = exon unless @exon_list[arm]
3   @exon_list[arm] = exon if exon.record.score > @exon_list[arm].record.score
4 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 initialized a parsing function is set up. Then, when each alignment is added, the id if the target sequence is parsed using the

```

SNP-1 A      cgcatttGcgcgYgcgataccggcgctKtgGaatatttcagcgaaggcgtg
SNP-1 B      cgcatttAcgcgYgcgataccggcgctKtgAgaatatttcagcgaaggcgtg
IWGSC-1A     cgcatttGcgcgCcgataccggcgctGtgGaatatttcagcgaaggcgtg
IWGSC-1B     cgcatttAcgcgCcgataccggcgctTtgGaatatttgc---gaaggcgtg
IWGSC-1D     c---atttGcgcgTgcgataccggcgctGtgGaatatttcagcgaaggcgtg

```

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

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
2     do | contig_name |
3         ret = contig_name[0,2]
4         return ret
5     end

```

To ensure that the indels between homoeologues don't produce spurious mismatches a local alignment is produced with MAFFT (Figure 2.10). The arguments used are the recommended 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::PolyplloidTools::SNP` has the method `aligned_sequences` which execute MAFFT for the best hit on each chromosome to the marker. The first time it is invoked and 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 which 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 identify 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).
2. Then each position in the mask is iterated base per base (Listing 2.11, line 7).

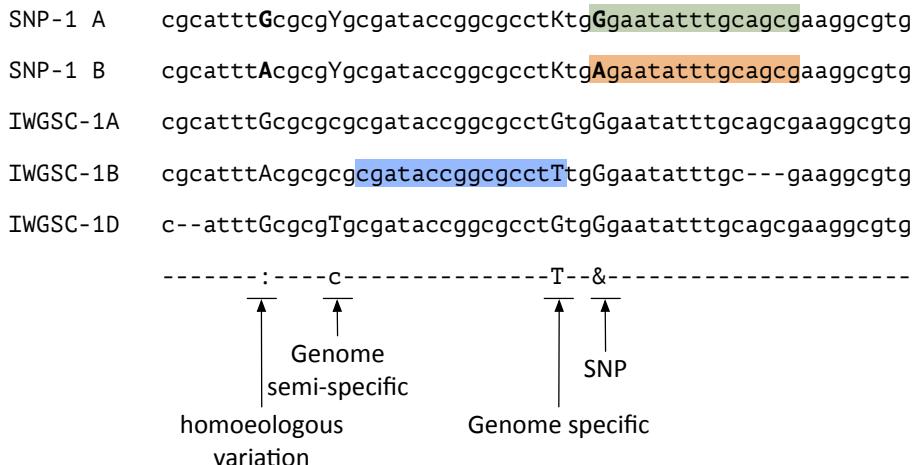


Figure 2.11: Alignment with mask and primer candidates. The green and light red boxes highlight the allele specific primers. The blue box highlights a genome specific primer.

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 doesn't 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 initialization 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 ploidy.
7. At the position of the SNP, the special symbols are setup (lines 23-30)
 - (a) By default, the SNP position is labelled as & (line 24).

- (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. This is a reasonable size for KASP markers, as the technology doesn't 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 **--primer3_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 semis-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

```

1 def mask_aligned_chromosomal_snp(chromosome)
2   names = exon_sequences.keys
3   parentals = parental_sequences.keys
4   local_pos_in_gene = aligned_snp_position
5   masked_snps = aligned_sequences[chromosome].downcase
6   i = 0
7   while i < masked_snps.size
8     different = cov = from_group = Count = 0
9     names.each do | chr |
10       if aligned_sequences[chr] and aligned_sequences[chr]
11         [i] != '-'
12       cov += 1
13       nCount += 1 if aligned_sequences[chr][i] == 'N' or
14         aligned_sequences[chr][i] == 'n'
15       from_group += 1 if chr[0] == chromosome_group
16       if chr != chromosome
17         different += 1 if masked_snps[i].upcase !=
18           aligned_sequences[chr][i].upcase
19       end
20     end
21     masked_snps[i] = '-' if different == 0 or if cov == 1
22       or nCount > 0
23     masked_snps[i] = '*' if cov == 0
24     expected_snps = names.size - 1
25     masked_snps[i] = masked_snps[i].upcase if different ==
26       expected_snps and from_group == genomes_count
27     if i == local_pos_in_gene
28       masked_snps[i] = '&'
29     bases = ''
30     names.each do | chr | { bases << aligned_sequences[
31       chr][i] if aligned_sequences[chr] and
32         aligned_sequences[chr][i] != '-' }
33     code_reference = 'n'
34     code_reference = Bio::NucleicAcid.to_IUAPC(bases)
35       unless bases == ''
36     masked_snps[i] = ':' if Bio::NucleicAcid.is_valid(
37       code_reference, original) and Bio::NucleicAcid.
38       is_valid(code_reference, snp)
39     end
40   i += 1
41 end
42 end
43 masked_snps
44 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 prioritize 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: Initialization of the `Bio::DB::Primer3::Primer3Record` class including the default score weights

```

1 def initialize
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-homoeologus. 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 secibd 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.

2.1.1 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 on-line 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.

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 visualization 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.

2.2 Applications of PolyMarker

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

2.2.1 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 using the target chromosome using the genetic map provided in Wang et al. (2014). Genes without a genetic position were aligned to scaffolds sorted by chromosome from the International Wheat Genome

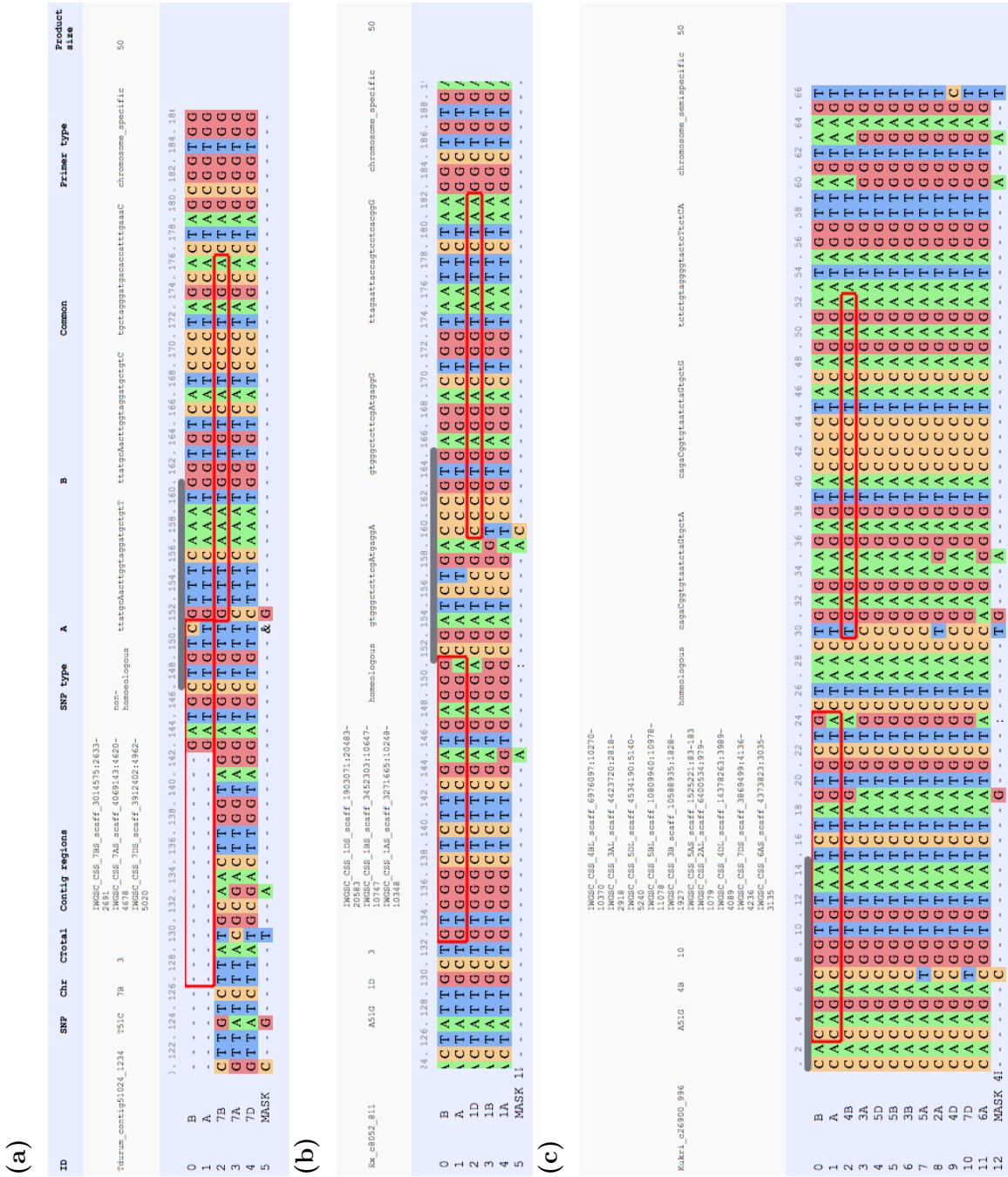


Figure 2.12: Examples outputs from the PolyMarker website. (a) The primer triplet is genome specific. The original marker sequence had the SNP near the begining of the template, but PolyMarker used the genomic reference to complement the sequence. (b) An specific primer, but the SNP is located on the 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

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 markers had been taken 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 `exonrate` (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 Axiom array.

2.2.2 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 crossed to fix the mutations. The second generation is called M_2 , and so on. With each generation the originally heterozygous mutations get fixed

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-specific	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

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_2 , 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) were also validated. Genome-specific primers 172 and 80 SNP assays on 19 and 8 M_4 Cadenza and Kronos lines respectively. Of those, 71(85.5%) Kronos and 147(88.8%) of the Cadenza primers were valid assays, consistent with the pilot study (Tables A.1 and A.2).

2.3 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 analysis. On each of the following projects, PolyMarker has been adapted to design primers in species where KASP hasn't been used before, the primers are used for

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%

regular PCR amplification, or the use of KASP is not the conventional SNP calling.

2.3.1 Deletions on a mutant population

On some of the TILLING mutant lines long deletions 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 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.

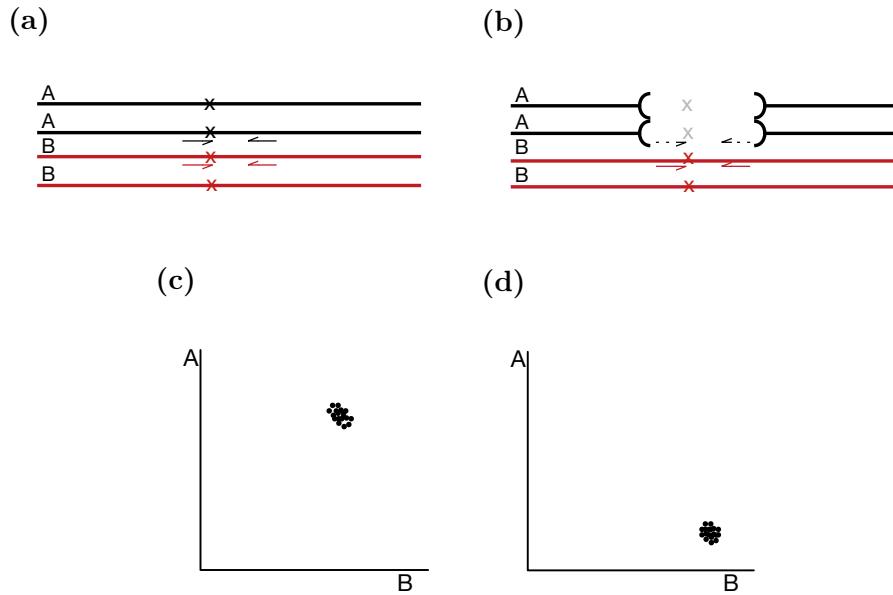


Figure 2.13: KASP assays to validate homozygous deletions. (a) Primer positions for wildtype. Red and black indicate the A and B genome respectively. Primers are indicated by arrows with the target homoeologous SNP marked by an "X"(b) Primer positions on homozygous deletion on M_4 (c) Heterozygous amplification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

Listing 2.8: Score values to select semi-specific 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 didn't 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.

2.3.2 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 of chromosomes. For PolyMarker it can be treated as 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. (2011). 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, previous method to genotype of PST was SSR markers.

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.

Marker	Deletion	chr	cM	1	2	3	4	5	6	7	8	9	10	11	12	C	C	C	Result
5BL_2297308_Cadenza0423_12664_C12664T	-	5B	4.551	X	X	-	X	X	X	X	X	X	-	X	Y	Y	Y	HOM Mutation	
5BL_10812849_Cadenza0423_5664_G6664T	-	5B	38.769	X	X	-	X	X	X	X	X	X	-	X	Y	Y	Y	HOM Mutation	
5BL_10825062_Cadenza0423_7917_G7917A	-	5B	38.769	X	X	-	X	X	X	X	X	X	-	X	Y	Y	Y	HOM Mutation	
IWGSC_CSS_5BL_scaff_10847976_27068-27231	+	5B	38.769	X	X	-	X	X	X	X	X	X	-	X	H	H	H	Hom Deletion	
IWGSC_CSS_5BL_scaff_10847976_28118-28674	+	5B	38.769	X	X	-	X	X	X	X	X	X	-	X	H	H	H	Hom Deletion	
5BL_1083722_Cadenza0423_4616_G4616A	-	5B	39.905	X	X	-	X	X	X	X	X	X	-	X	Y	Y	Y	HOM Mutation	
5BL_10891320_Cadenza0423_18847_C18847T	-	5B	45.594	Y	Y	-	Y	H	X	X	Y	H	-	H	Y	Y	Y	HET Mutation	

Table 2.5: PolyMarker used to genotype PST. The X and Y represent 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.

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

2.4 Discussion

PolyMarker is a tool that was born as part of the validation of the SNPs found in Chapter 3. Originally, 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 figured out the 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 and it even allowed the novel use of KASP assays to validate long deletions in polyploids.

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 won't 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 time, 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 a blast search 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 constraints for the 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 requires 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). On personal communications in conferences

Look at
<http://www.ncbi.nlm.nih.gov/p>
to get
some
way to
compare
SRR vs
SNP
markers

I had found out that LGC uses PolyMarker to design primers and that 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.

Overall, PolyMarker provides an useful resource to the wheat community, as the primer design process is now streamlined. As new references of wheat come available, PolyMarker should be updated to work with pseudomolecules and the web interface updated accordingly. The source code of PolyMarker is open source and available on <https://github.com/TGAC/bioruby-polyplloid-tools>.

Chapter 3

Genetic map of *Yr15* with RNA-Seq

3.1 Introduction

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*, the fungi responsible of yellow rust. A source of resistance genes is are introgressions from other species, such as *Triticum dicoccides*. 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). On this chapter the NIL for the *Yr15* locus is used to produce a mapping population to improve diagnostic markers.

3.1.1 Segregation on F_2 populations

Line selection can be done with molecular markers that can be used to test if certain allele is present in a line, without the need to do a phenotype. To find which regions are linked to a trait the use of F_2 mapping populations is a common practice. The population is produced by crossing two homozygous parents (P_1 and P_2) with different alleles, A/A (dominant) and a/a (recessive). When the trait is dominant and has a mendelian segregation, the F_1 population show the dominant trait, as it has a copy of each allele (A/a). The F_1 is then self-crossed to and the population segregates with a ration 1:2:1, dominant:heterozygous:recessive

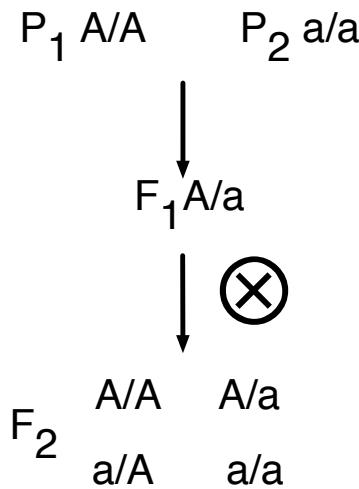


Figure 3.1: The cross of two homozygous parents, P_1 and P_2 , with a dominant and a recessive allele of a gene produce an heterozygous F_1 . The F_1 crossed with itself produce a segregating F_2 population with a 1:2:1 ratio (A/A:A/a:a/a). The upper and lower cases represent dominant and recessive alleles

respectively. This generates a population with a phenotype ratio of 3:1 (dominant:recessive), since the effect of the recessive allele is masked by the dominant gene (Van Ooijen and Jansen 2013; Figure 3.1).

3.1.2 SNP calling

Bulk Segregant Analysis (BSA) consists on pooling the DNA of individuals with contrasting phenotypes (Michelmore et al., 1991) on a segregating population. The bulks show as heterozygous except for the region that is linked to the trait of interest. This approach can be used to identify SNPs using High Throughput Sequencing, such as: exome capture (Hodges et al., 2007), RNA-Seq (Pickrell et al., 2010), whole genome resequencing (Schneeberger et al., 2009), among others.

To Call for SNPs from RNA-Seq a reference transcriptome is used as target to align the reads. The Bulk Frequency Ratio (BFR) methodology can work on organisms that have more than one pseudo genome with not all the genes, homoeologues or paralogues, characterised independently; it works with a single reference collapsing similar regions. The UniGenes database, from NCBI, contains the genes of each species with all the variations of each gene automatically collapsed and represented with the longest cDNA (Pontius et al., 2002). The UCW genes 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 ho-

moeologues aren't merged in a single model, a possible side effect of the UniGene pipeline.

Homoeologous variants, as exemplified by the G>T variant at position 181; K in consensus (Figure 3.2), will produce the same ambiguity code for both parental consensus sequences and can therefore be excluded. Real allelic SNPs between the parental genotypes, exemplified by the G>A variant at position 184; R in consensus, are distinguished by the presence in one, but not the other parental consensus sequence. The allelic SNPs are then examined further with the alignments of the bulks to identified 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, in the example the the susceptible bulk has an SNP index of $1/8 = 0.125$ and $6/8 = 0.75$ for the resistant bulk (Takagi et al., 2013b). traditional The BFR are then calculated by dividing the SNP Index of sample containing the target phenotype (resistance) over the sample without the trait (susceptible), on the example is $0.75/0.125 = 6$. 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.

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 genetic maps previosly published, such as the genetic map 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 percentage of identity (over 98%), to avoid them being assigned to an homoeologue or parologue region in a different chromosome. The use of genetic maps to sort genomic sequence is frequently used to produce pseudo-chromosomes on genome wide projects, usually with ad-hoc tools (Tang et al., 2015). Since the CSS assembly is quite fragmented the genetic maps don't have enough resolution to produce a pseudomolecule, however it is enough to sort the scaffolds in bins when several markers

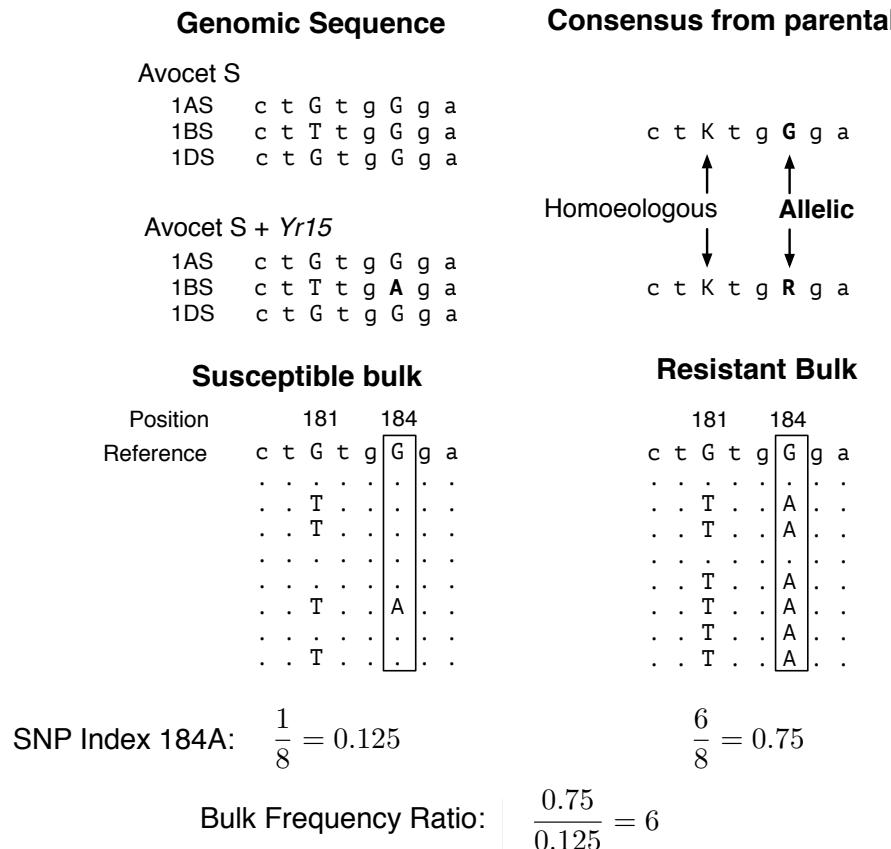


Figure 3.2: BFR formula. Illustration of a non-informative homoeologous SNP (G181T) present in both parental lines, and an informative allelic SNP (G184A), only present in the resistant progenitor Avocet S + Yr15. The consensus sequences from the parental genotypes include this information in the form 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 at those positions. 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).

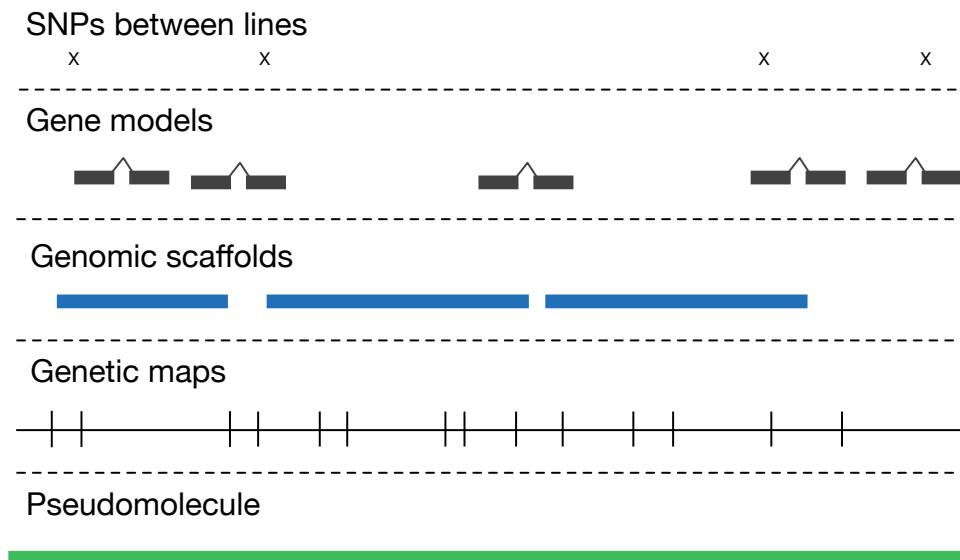


Figure 3.3: Layers of information to do *In Silico* mapping. SNPs are called from gene models. The genes and markers from genetic maps are aligned to scaffolds. The order of the markers in a genetic map can be used to sort the scaffolds.

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 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 1.7.

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

To do:
section talking about genetic map.

To do:
Mi-crosatellites vs SNP markers.

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 were sown and tissue was collected, before the fungal inoculation to avoid the effect of the response on the

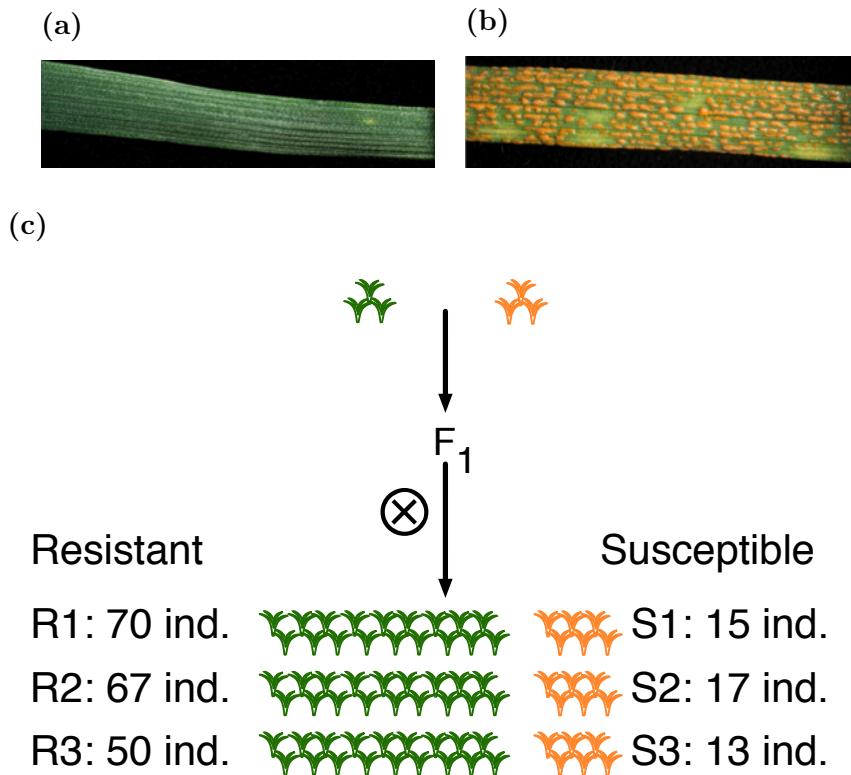


Figure 3.4: Avocet + *Yr15* *F₂* mapping population. Response of (a) Avocet + *Yr15* and (b) Avocet when inoculated with *Puccinia striiformis* f. sp. *tritici* at the three leaf stage. (c) The phenotype of the *F₂* population was used to produce 6 bulks, 3 resistant and 2 susceptible. The RNA was pooled in bulks accordingly. Adapted from (Ramirez-Gonzalez et al., 2015c)

gene expression. The plants 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 on an *F₂* population is 3:1 (resistant:susceptible), since *Yr15* is a dominant gene. From the 232 plants in the *F₂* 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 assays (i.e. plants scored as resistant without the *Yr15* locus). For this study we extracted DNA from individual plants in the *F₂* 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 + <i>Yr15</i>	N/A	4	3.99

3.3 Sequencing and mapping

RNA-Seq was used to avoid sequencing the non-coding regions and reduce the search space. The sequencing of the bulks and the parents were done on a single Illumina Hi-Seq2000 each. The bulks were multiplexed and sequenced on a third of a lane each, as shown on Table 3.1. To ensure that the quality of the sequencing was good, **fastqc-0.10** (Babraham Bioinformatics, 2012) was run with its default parameters in each one of the fastq files. The GC content was around 52% in all the samples (Appendix B.2), which is 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 the base 80 across the samples (Appendix B.1).

When the analysis was started, the draft genome and the corresponding annotation where not released yet, hence gene models were used. 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 provided showed that a few genes were overly expressed, however we still have 22,107 and 36,808 genes, on the Unigenes and the UCW gene set respectively, with a coverage greater than 20x in the progenitor with *Yr15*. Both gene sets performed similarly in terms of the percentage of genes with reads and percentage of aligned reads. For AVS and *Yr15*, the percentage of genes with a coverage of at least 20x is 45% and 39% respectively across both references (Figure 3.5a). Since each individual bulk has a 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

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

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

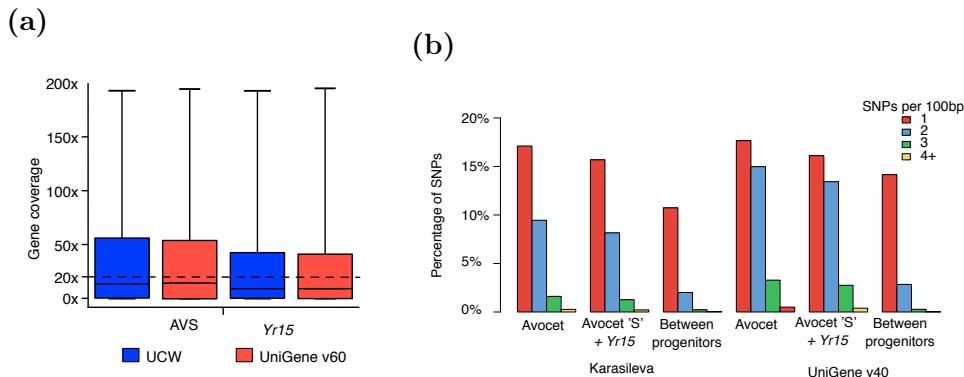


Figure 3.5: Average coverage and SNPs between progenitors. (a) Box plot distribution of the gene coverage of the parent reads (AVS and *Yr15*) across the UCW (blue) and the UniGene (red) gene models. The dashed line represents the 209 minimum coverage required for SNP calling. The full line represents the average coverage across all gene models. (b) Percentage of genes exhibiting SNPs across references. The number of SNPs 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)

susceptible ($S_1 + S_2 + S_3$) and resistant bulks ($R_1 + R_2 + R_3$). 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.

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 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 references can be explained by the fact that the UniGenes have homoeologues can be represented as a single sequence, as opposed to the UCW set which are homoeologue-specific, improving the mapping to the correct homoeologue in the genes from the UCW set over the UniGenes.

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

SNPs per 100bp	UCW			UniGene v60		
	AVS	AVS+ <i>Yr15</i>	Between progenitors	AVS	AVS+ <i>Yr15</i>	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%

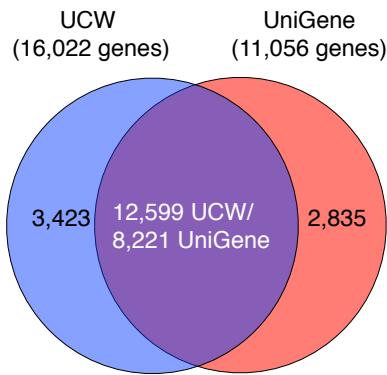


Figure 3.6: Gene models with putative SNPs in common between the UCW and UniGenes reference. The intersection represents the genes that are common in both sets. Adapted from Ramirez-Gonzalez et al. (2015c)

Both gene sets were done 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 an 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 1.6.1; Figure 3.2). To focus the analysis on informative SNPs, the common varietal SNPs and variations between homoeologues were removed by finding the cases when the consensus call on both progenitors is the same. The SNPs that are unique to a single parental were 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 an *F*₂ mapping population expects to have < 1% of the genetic background segregating. The both sets of gene models were aligned with BLAT (Kent, 2002) to the Chinese Spring Chromosome arm sur-

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

vey sequence (CSS; 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 (Figure 3.10a), suggesting that the Avocet S (JIC, UK) used as parent in the F_2 is different to the Avocet S used for the *Yr15* NIL development (University of Sydney, Australia).

To confirm that the Avocet S seed stocks from JIC are distinct to the stocks in Sydney DNA from both stocks was procured and compared with the iSelect 90k wheat SNP chip. Between two independent Avocet S seeds from JIC only 58 out of 71,972 (0.08%) valid assays were polymorphic. Nonetheless, ther are over 5,000 ($> 7.5\%$) assays with polymorphisms between JIC-Avocet S and Avocet S from Sydney. The different was not expected originally, but considering that the Avocet S 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 find the SNPs enriched on each bulk and hence linked to the phenotype, variations from *Yr15* to resistance and from AVS to susceptibility in the segregating population. Across individual bulks, it was possible to score between 15,261 (24.5%) to 31,891(48.0%) SNPs 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.

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 + 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+R2+R3 Yr15	S1+S2+S3/ S1+S2+S3/ 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 ^a (3.48%)	860/24,026 (3.58%)	1,505/40,496 (3.72%)
		UniGene v60 (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 (3.33%)	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 (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%)	142/1,377 (10.86%)	111/1,101 (12.65%)	147/1,162 (10.56%)	149/1,411 (12.61%)	167/1,324 (12.61%)	163/1,478 (11.03%)	194/1,370 (14.16%)	207/1,765 (11.73%)
		UniGene v60 (13.56%)	58/527 (11.01%)	101/1,017 (9.93%)	81/720 (11.25%)	105/775 (13.55%)	84/867 (9.65%)	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 (25.59%)	118/646 (17.43%)	70/409 (18.37%)	123/577 (24.54%)	85/494 (15.24%)	145/673 (20.38%)	98/563 (17.41%)	168/768 (21.56%)	122/665 (18.35%)
		UniGene v60 (25.98%)	16/186 (13.98%)	83/390 (21.28%)	54/294 (18.71%)	93/379 (28.47%)	48/315 (24.54%)	107/315 (24.13%)	66/679 (18.69%)	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 (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 (24.13%)	127/519 (24.47%)	29/266 (10.90%)
5	UCW	69/202 (34.16%)	19/108 (17.53%)	95/416 (22.84%)	33/138 (23.91%)	105/416 (27.12%)	44/246 (16.08%)	134/539 (26.62%)	53/277 (16.00%)	140/580 (23.14%)	42/222 (18.92%)
		UniGene v60 (35.58%)	11/70 (15.71%)	76/337 (22.55%)	14/102 (13.73%)	70/228 (30.70%)	20/112 (17.86%)	127/477 (25.71%)	28/175 (15.75%)	140/640 (24.14%)	21/178 (11.80%)
6	UCW	65/179 (34.31%)	12/85 (14.12%)	86/380 (22.63%)	22/98 (22.45%)	87/299 (29.10%)	11/94 (12.62%)	122/429 (16.62%)	21/130 (16.00%)	126/514 (24.14%)	29/165 (18.92%)
		UniGene v60 (37.75%)	57/151 (14.58%)	7/48 (10.81%)	73/300 (24.33%)	6/71 (8.45%)	65/191 (34.03%)	13/84 (15.48%)	98/358 (27.37%)	23/146 (16.39%)	118/469 (25.16%)
7	UCW	58/161 (36.02%)	11/73 (15.0%)	77/340 (22.65%)	13/74 (17.57%)	73/248 (29.44%)	7/69 (10.14%)	122/429 (29.52%)	20/111 (18.02%)	114/468 (24.36%)	22/143 (15.38%)
		UniGene v60 (42.42%)	56/132 (4.37%)	68/273 (25.10%)	5/58 (10.00%)	60/171 (35.09%)	9/64 (14.06%)	94/334 (16.36%)	18/103 (15.73%)	113/412 (27.43%)	16/143 (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%)	104/393 (28.97%)	17/102 (16.67%)	108/429 (25.17%)	16/119 (13.45%)
		UniGene v60 (43.65%)	55/126 (3.33%)	64/255 (9.09%)	5/50 (25.41%)	55/150 (10.87%)	9/55 (16.67%)	91/313 (16.36%)	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 (45.30%)	53/117 (1.33%)	62/244 (2.41%)	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 (47.62%)	50/105 (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%)

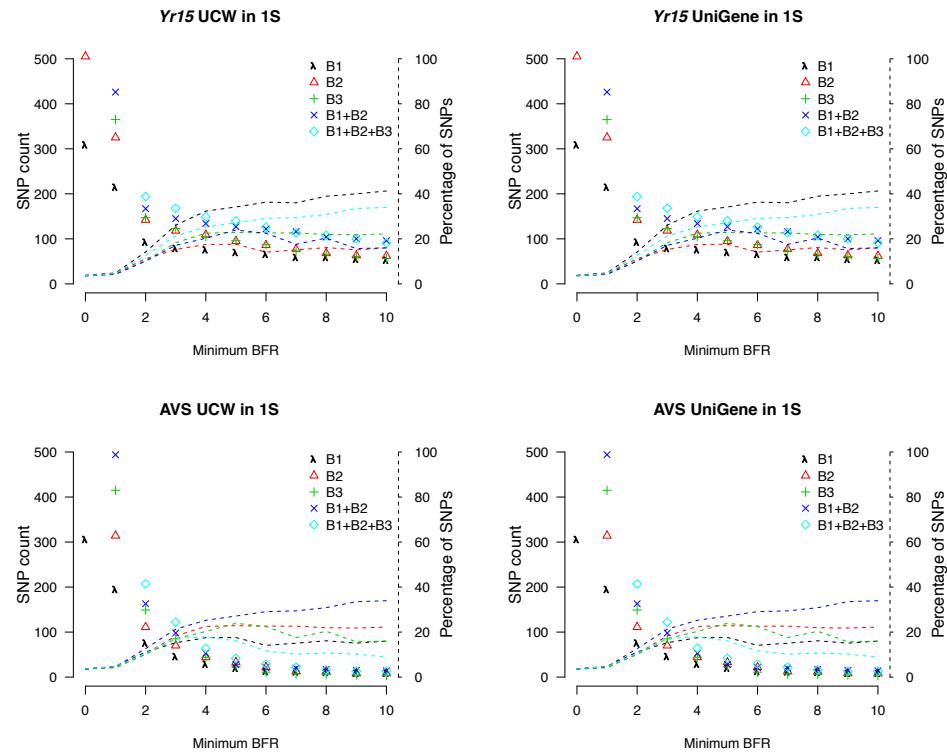


Figure 3.7: Effect of BFR threshold on the number of SNPs across the short arm of chromosome group 1. Figure previously published in Ramirez-Gonzalez et al. (2015c).

When increasing the minimum BFR threshold, enrichment of SNPs was observed in the short arm of the group 1 chromosomes (1S). Without taking in 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 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 $BFR > 6$.

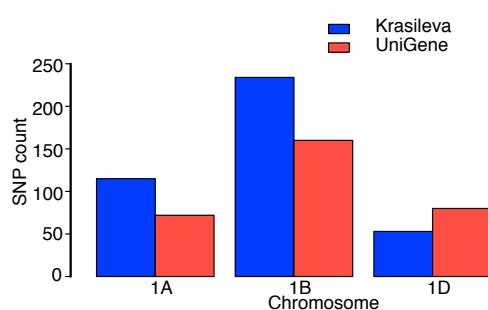


Figure 3.8: Location of SNPs with $BFR > 6$ according to the best alignment of the UniGene (red) and UCW (blue) gene models to the flow-sorted group 1 chromosomes from the Chinese Spring Survey sequence (CSS) (Mayer et al., 2014). Figure adapted from Ramirez-Gonzalez et al. (2015c).

3.6 *In silico* mapping

From the mapped SNPs with a $BFR > 6$, 872 of 1470 ($\sim 60\%$) 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 the 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). This 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 SNPs 40,266 SNP markers published at the time when this analysis was done (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-enable genetic approaches (James et al., 2013) have shown

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 Chromosome	CSS assemblies						Genetically mapped									
	UCW gene models		UniGene v60		UCW gene models		UniGene v60		SNPs		Genes					
	SNPs	Genes	SNPs	Genes	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%

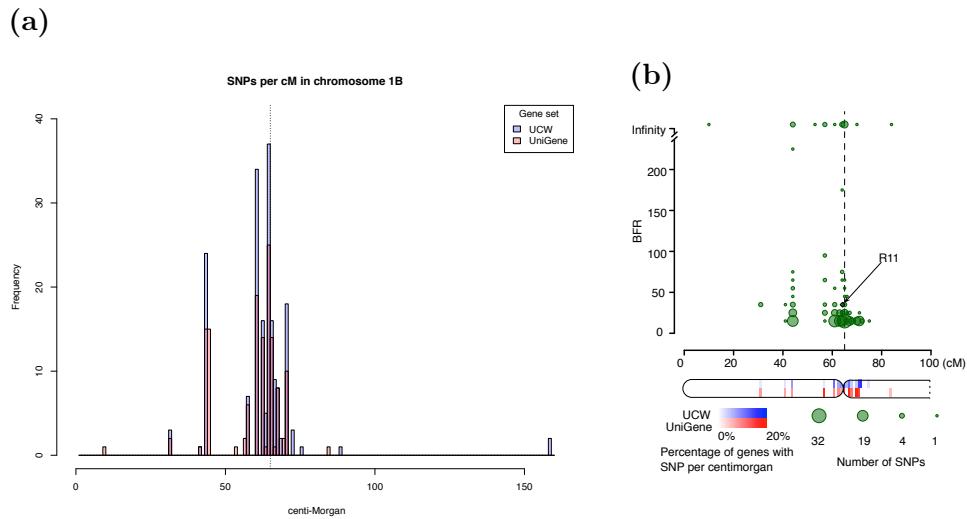


Figure 3.9: *In silico* location of SNPs with $BFR > 6$. (a) Number of SNPs with $BFR > 6$ per cM in chromosome 1B. (b) BFRs of mapped genes with SNPs on chromosome 1B. The area of the circle represents the number of SNPs clustered by location (windows size: 10 cM) and BFR (window size: 5cM). R11 is 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).

smooth curves with a defined peak in the region linked to the studied 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; like the biases induced by the differential expression, the fragmented reference sequence with scaffolds that are not long enough to go across genetic positions. Since there are several SNPs with a high BFR and the genetic map is not enough to locate a single region linked to *Yr15*, multiple criteria was needed to prioritise SNPs that were more likely to yield on successful genetic markers.

3.7 Assay selection

Three independent criteria were use to prioritize 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. This is to be 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.

With the multiple criteria the number of genes with a putative SNP went down from $> 27,000$ to just 175; 77 and 98 from the UniGene and

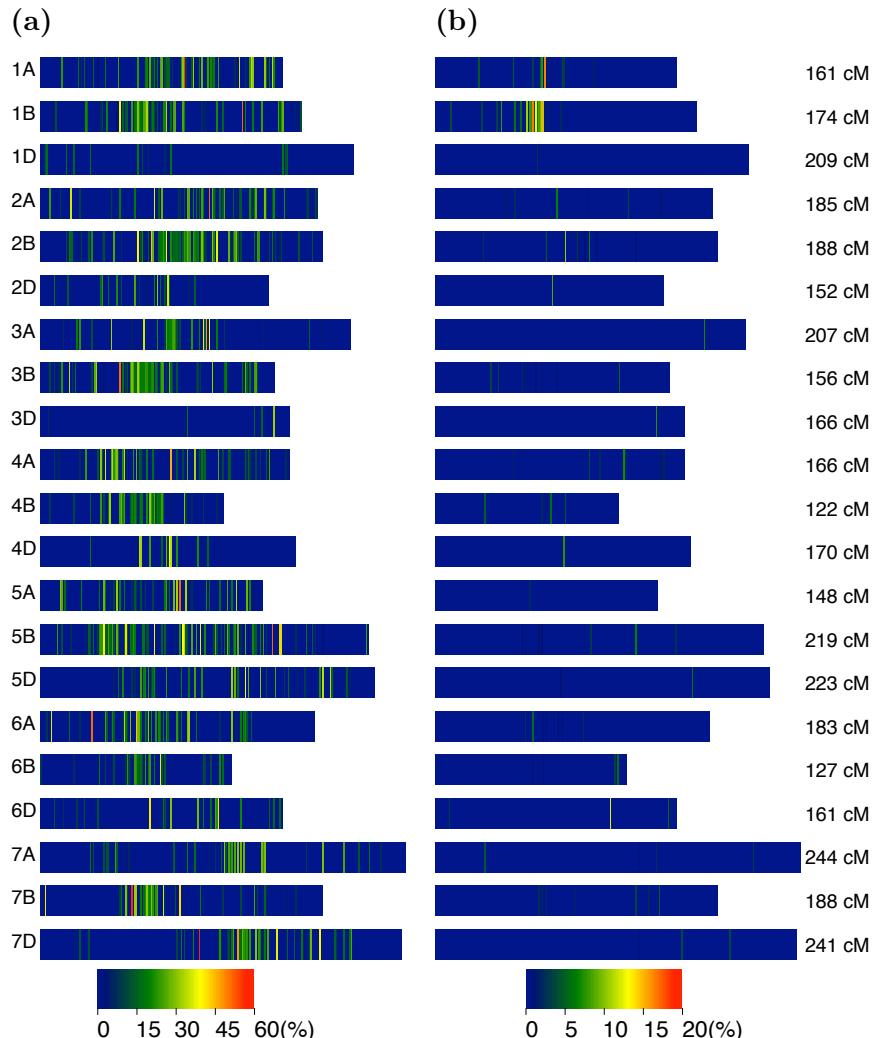


Figure 3.10: Genetic location of genes with SNPs between AVS and Yr15. The colour scale indicates the percentage of genes with SNPs per centi-Morgan (cM) across the 21 wheat chromosomes. The location of the genes was determined by the best alignment to the CSS scaffolds, and the location of these was 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

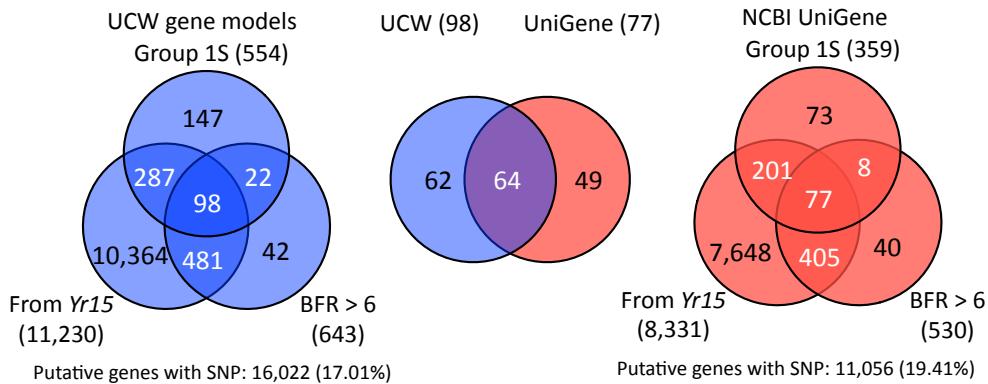


Figure 3.11: Selection criteria for marker design. Venn diagrams based on the three selection criteria (SNP in the short arm of chromosome group 1; SNP has a $BFR > 6$; and SNP is from the *Yr15* parent) for the UCW (blue) and UniGene (red) gene models. The centre diagram shows the intersection between 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)

UCW gene sets respectively. The selected genes from both references were aligned between references, as they come from independent sources an overlap in the selection between them is expected and, as expected, around half of the genes between gene sets overlap (Figure 3.11). The 50 SNPs with the highest BFRs, out of the 175 genes, were selected for validation, 15 of them were redundant between references, resulting on 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 as the number of SNPs with $BFR = \infty$ should drop in the mixes, as the improved coverage should reduce the instances where the absence of an allele is because of 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(marker R5). On some cases a SNP are missing in an individual bulk, but present in the rest of them and in the mixes (marker R8). The main reason affecting the scoring is the coverage in the sample for each particular gene, hence an strategy with a consistent coverage would be preferred for this kind of analysis. Previous studies have shown that a

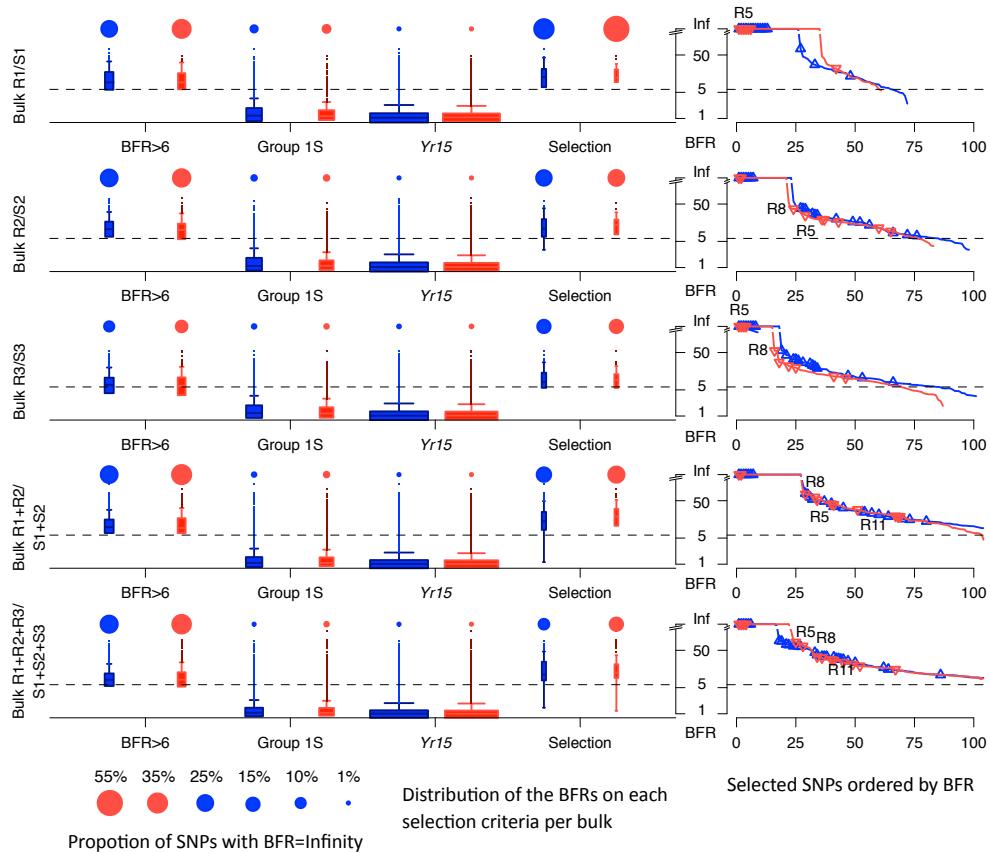


Figure 3.12: BFRs of selected SNPs across the individual bulks and in silico mixes (UCW, red; UniGene, blue). The dotted line represents the BFR threshold of 6 (logarithmic scale). Left: Distribution of the BFRs for each selection criteria and the selected SNPs for validation. The circles on the top 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.

Table 3.8: Number of genes (and SNPs) with a unique hit (> 99% sequence identity) to a single wheat survey 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 + UniGene	Unique	8,970	1,990	524	353	5.84%	17.74%
	Total	14,508	3,272	872	576	6.01%	17.60%
	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 + UniGene	Unique	66,539	15,283	825	620	1.24%	4.06%
	Total	118,688	27,078	1,582	1,173	1.33%	4.33%
	Percentage	56.06%	56.44%	52.15%	52.86%		

coverage of $< 5x$ is enough to call for 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, specially 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 > 99% sequence identity to a single CSS scaffold (Table 3.8). This is likely because there is no unique homoeologue in the gene models, leading to reads, from two different homoeologues, mapping to the same region. To reduce the number of spurious SNPs we used IUAPC ambiguity codes (Section 1.6.1, Cornish-Bowden (1985)) when two different alleles were observed. This had as 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

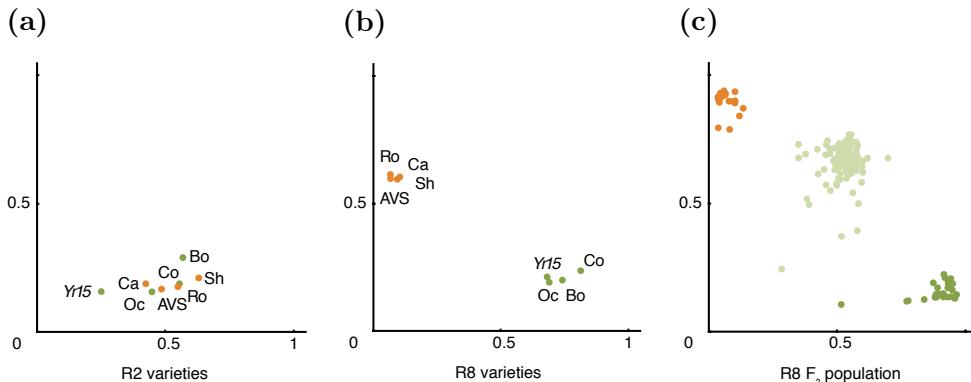


Figure 3.13: KASP output from the wheat variety panel with (Ochre, Boston, Cortez) and without (Robigus, Cadenza and Shamrock) *Yr15*. Marker R2 (a) is monomorphic while R8 (b) is polymorphic between varieties known to carry the gene. Marker R8 results for the F₂ population (c) showing three distinct 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).

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 has to be reached in line with the objectives and available resources for a particular study.

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 homoeologous 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 to *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

Table 3.9: Primer details for the markers to validate.

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. diccoccoides* instead of *Yr15*. On the test panel, 28 (80%) SNPs were polymorphic across the parents and three of them were diagnostic to *yr15* (R5, R8, R33). From the five homoeologous SNPs, three of them were monomorphic and 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 fall 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 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).
- A marker based on barley-wheat synteny (R43) which met the selection criteria, but wasn't 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 SSRs, 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 on an *F*₂ population of 196 individuals, as 392 gametes provide a resolution of 0.26cM. Despite the fact that none of the selected markers have perfect linkage to *Yr15*, the produce genetic map is an improvement in the resolution of the map for the locus and it enables the shift to SNP markers from microsatellites, which

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	<i>Yr15+</i>		<i>Yr15-</i>		comment
				Yr15 Ochre	Yr15 Shamrock	Yr15 Robigus	Yr15 Cadenza	
R1	UCW	UCW_Tk-k55_contig_8830;tt-k21_contig_10204	C341G	A	H	A	A	-
R2	UniGene v60	gnl UGITa#S13126619	C491T	B	B	B	B	Yes
R3	UCW	contig95240	C220G	H	B	B	B	No
R4	UCW	contig105384	C1227T	A	B	B	B	Yes
R5	UniGene v60	gnl UGITa#S58861868	A214G	A	A	B	B	Yes
R6	UCW	KukriC706_1	T2979C	A	H	B	B	Yes
R7	UniGene v60	gnl UGITa#S37932863	C281T	H	A	A	B	No
R8	UniGene v60	gnl UGITa#S58863387	T241C	B	B	A	A	Yes
R9	UniGene v60	gnl UGITa#S588892239	C303T	H	B	B	B	No
R10	UCW	UCW_Tk-e63_contig_79829	C207T	H	A	B	B	Yes
R11	UCW	UCW_Tk-k45_contig_39011	C726T	A	A	H	-	Yes
R12	UCW	contig50308	G587A	-	H	H	B	Yes
R14	UniGene v60	gnl UGITa#S44692929	C549T	A	A	A	A	Yes
R15	UCW	UCW_Tk-k51_contig_2344;tt-k55_contig_2091	T686G	A	A	A	A	-
R16	UniGene v60	gnl UGITa#S17898149	G227A	A	B	A	B	Yes
R17	UCW	CL3339Contig1	T509C	H	H	H	H	No
R19	UCW	UCW_Tk-e21_contig_8407;tt-k61_contig_5972	C1405T	A	B	B	B	Yes
R20	UCW	UCW_Tk-k21_contig_8407;tt-k61_contig_5972	T1102C	A	B	B	B	Yes
R21	UCW	UCW_Tk-k31_contig_53804;tt-k41_contig_31582	G1810T	H	B	B	B	Yes
R22	UCW	UCW_Tk-k31_contig_14966	T408C	A	A	A	A	Yes
R23	UCW	UCW_Tk-k31_contig_12731;tt-k55_contig_13077;tt-k61_contig_18734	C507T	A	H	H	-	Yes
R24	UCW	UCW_Tk-k55_contig_8830;tt-k21_contig_10204	T3005G	H	H	B	B	Yes
R25	UCW	UCW_Tk-k63_contig_79829	G184A	A	A	A	A	No
R26	UCW	UCW_Tk-k21_contig_3794	C702T	H	A	B	B	Yes
R28	UCW	KukriC701_1	T1053C	A	A	B	B	Yes
R29	UCW	UCW_Tk-k55_contig_8640;tt-k41_contig_8875	G783A	H	A	B	B	Yes
R30	UCW	UCW_Tk-k55_contig_8830;tt-k21_contig_10204	T2184A	A	B	A	B	Yes
R31	UCW	UCW_Tk-k45_contig_22098	G683T	A	B	B	A	Yes
R32	UCW	UCW_Tk-k21_contig_33188;tt-k25_contig_30647	C596A	H	A	A	A	No
R33	UniGene v60	gnl UGITa#S58861868	G486T	A	A	B	B	Yes
R34	UCW	UCW_Tk-k31_contig_34099	G1713A	H	A	B	B	No
R35	UniGene v60	gnl UGITa#S58900202	T889C	A	B	B	B	Yes
R36	UCW	UCW_Tk-k55_contig_8830;tt-k21_contig_10204	T2349C	H	H	-	H	Yes
R37	UCW	UCW_Tk-k31_contig_34099	C846T	B	B	B	B	No
R38	UniGene v60	gnl UGITa#S588840501	T179G	B	B	B	B	No
R40	UCW	UCW_Tk-k31_contig_34099	C846T	A	H	B	B	Yes
R43	UniGene v60	gnl UGITa#S588843705	G268A	A	B	-	B	Yes

Linked *Yr15*
Polymerphic
SNP
Cadenza
Robigus
Ochre
Shamrock
Yr15+/-

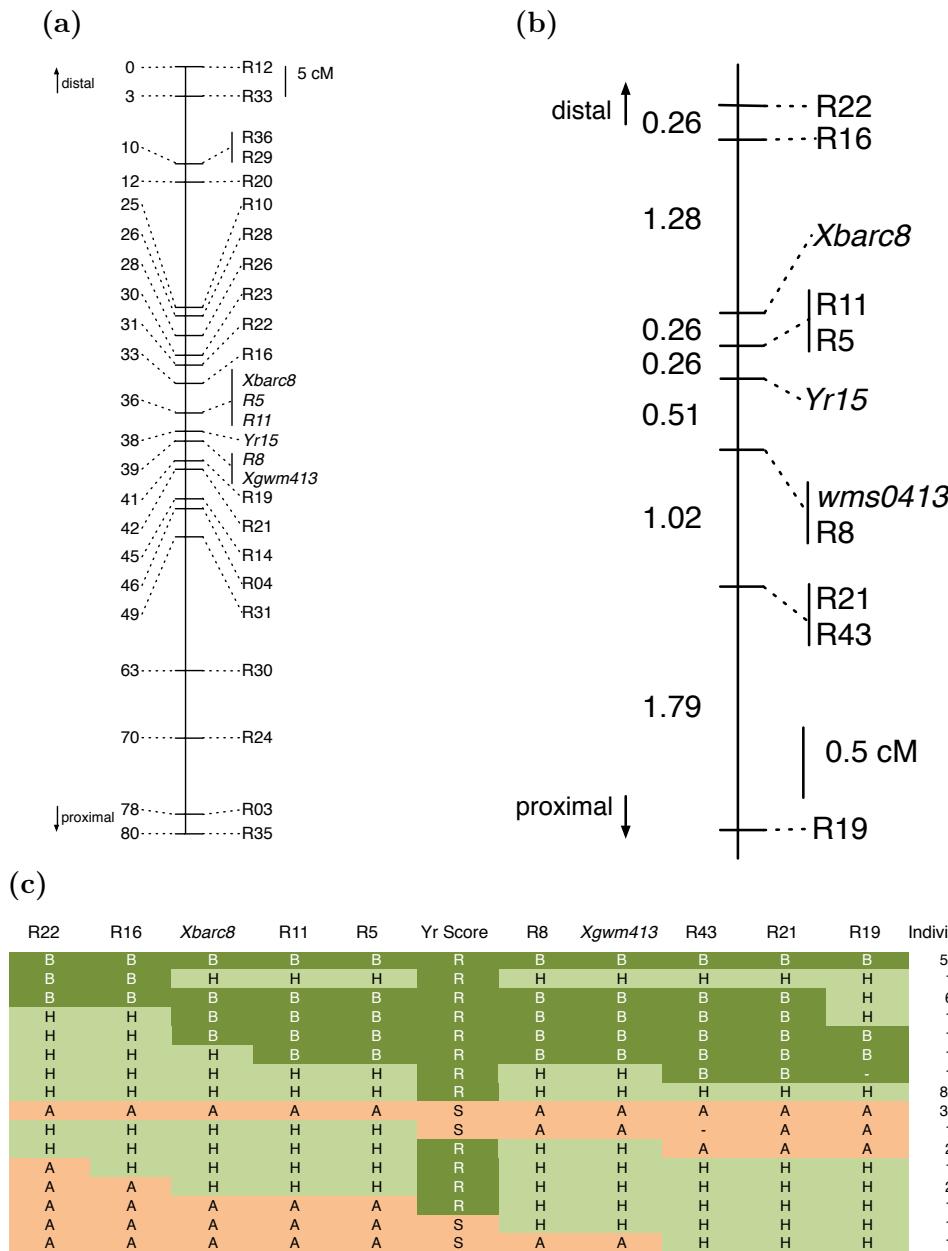


Figure 3.14: Genetic maps for *Yr15*. (a) Genetic map of the test panel from 50 individuals. (b) Genetic map from 196 individuals from the full population only with the 8 markers previously identified as closer to the *Yr15* locus. (c) Graphical genotype of the 196 *F₂* individuals used to develop the genetic map. The alleles are abbreviated according to their origin: A: AVS; B: *Yr15* and H: Heterozygous. Missing calls are indicated by a hyphen.

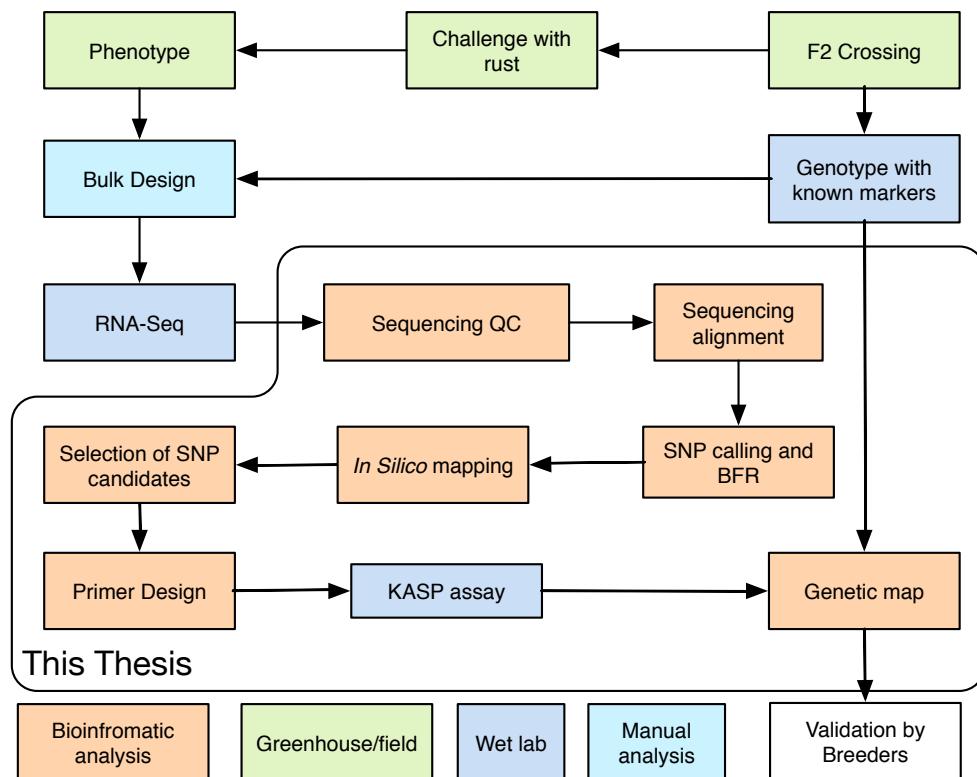


Figure 3.15: Steps used to go from the F_2 population to the genetic map.

has become the preferred marker system in MAS pipelines in breeding programmes.

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 demultiplexed with a tolerance of 1 mismatch in the barcode. Lanes 3 and 4 contained the parental sequences without a barcode. The

FastQ files were 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 were 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 followup 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 `minimum_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%), that allows for calling for a consensus even when more than one homoeologue is mapping to the same reference.

Snippet with submission of the alignments. However I haven't got access to the old cluster files.

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

```

1 def consensus_iuap(minimum_ratio_for_iuap_consensus)
2   minimum_ratio_for_iup_consensus
3   @consensus_iuap = self.ref_base.downcase
4   bases = self.bases
5   tmp = String.new
6   bases.each do |k,v|
7     if v/self.coverage > minimum_ratio_for_iup_consensus
8       tmp << k[0].to_s
9     end
10    if tmp.length > 0
11      @consensus_iuap = Bio::NucleicAcid.to_IUAPC(tmp)
12    end
13  end
14  @consensus_iuap.upcase
15 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.

```

1 def base_coverage
2   total = 0
3   @bases.each do |k,v|
4     total += v
5   end
6   total
7 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::BFRTTools::Container` was implemented to contain all the `BIO::DB::Sam` objects corresponding to the progenitors and the bulks. The class `Bio::BFRTTools::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 were the values of each region are calculated at once increases 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 to 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: S1âŠR1, Bulk 2: S2âŠR2 and Bulk 3: S3âŠR3); 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 sequence 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). To find the best hit for each query was kept 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; 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

```

1 def self.each_best_hit(text = '')
2   emptyHit = Bio::Blat::Report::Hit.new
3   emptyHit.score = 0
4   best_aln = Hash.new(emptyHit)
5   self.each_hit(text) do |hit|
6     current_score = hit.score
7     if current_score > best_aln[current_name].score
8       best_aln[current_name] = hit
9     end
10  end
11  best_aln.each_value { |val| yield val }
12 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 a location to an homoeologue in case that the correct scaffold is not in the genetic map. The genes were also align to the full CSS reference, to be able to locate the genes to a chromosome arm, even when it is not possible to assign a position in the genetic map and, to the cDNA 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.

Include code on how the coordinates where extracted, with the patch to the Ensembl package

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

```

1 class Bio::Blat::Report::Hit
2   def covered
3     match + mismatch
4   end
5   def query_percentage_covered
6     covered * 100.0 / query_len.to_f
7   end
8   def target_percentage_covered
9     covered * 100.0 / target_len.to_f
10  end
11 end

```

3.10.5 Primer design and KASP assays

The primer design for KASP were designed with PolyMarker as described in Chapter 2. The only difference with the default settings is that instead of using a template sequence, the sequence for each allele is calculated from the consensus of the alignments. The primers "were ordered from Sigma-Aldrich (Gillingham, UK), with primers carrying standardFAM or HEX compatible tails (FAM tail: 5' GAAGGTGACCAAGTTCATGCT 3'; HEX tail: 5' GAAGGTCGGAG TCAACGGATT3') with the target SNP at the 3' end. Primer mix was set up as recommended by LGC [46 µL dH₂O, 30 µL common primer (100 nM) and 12 µL of each tailed primer (100 nM)] (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 29 Kaspar mix and 0.056 µL primer mix]. PCR cycling was performed on a Eppendorf Mastercycler pro 384 using the following protocol: hotstart at 95 °C for 15 min, followed by ten touchdown cycles (95 °C for 20 s; touchdown 65 °C, ?1 °C per cycle, 25 s) then followed by 30 cycles of amplification (95 °C 10 s; 57 °C 60 s). 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).", as described in Ramirez-Gonzalez et al. (2015c).

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

Resequencing the ~ 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. The RNA and DNA extraction and the sequencing for this project was carried on before the beginning of my PhD (before October 2012). At that point exome capture was already established for genotyping humans (Ng et al., 2009), however the first exome capture on wheat was just recently published, with probes coming from unassembled 454 reads (Winfield et al., 2012), and a probe designed from transcripts (Henry et al., 2014) was not published after the analysis of this section was completed and validated. An even more targeted capture for resistance genes (RenSeq) was published while this study was executed (Jupe et al., 2013). On the other hand RNA-Seq was already tested for Bulk Segregant Analysis on tetraploid wheat (Trick et al., 2012). Hence, the decision of reducing the sequenced space with RNA-Seq was appropriate at the time (Figure 1.1). Unfortunately, one of the shortcomings of RNA-Seq used to call for SNPs is that the coverage is not uniform and the genes that have low expression don't have enough coverage to call for SNPs (Section 3.3). If a similar study is 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 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 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 Chinese Spring Chromosome arm survey sequence (CSS) assembly was being finished (Mayer et al., 2014); the longer scaffolds from Chapman et al. (2015) were not public yet and; the efforts to make a whole genome shotgun assembly were being planned independently by the International Wheat Genome Sequencing consortium (Pozniak, 2016) and TGAC (Clark, 2016). Because a contiguous assembly with the corresponding annotation wasn't available at the time of the analysis and the fact that the data available was from a transcriptome, 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 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 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 and were phased to distinguish between genomes and already published, over the MIPSv1 genes, still being refined from an initial approach lifting proteins from related organisms and a 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 were already submitted for publication (Figure 1.1; 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

Should I talk briefly about barley?. I would need to add a section before

are fragmented. Timely, a genetic map with $> 42,000$ markers was published (Wang et al., 2014). I was able to use it to locate several CSS scaffolds before the assembly was published, as I collaborated in the project. 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 they enabled the assignment of the genes to a chromosome.

The original expectation was to have a NIL for the BSA, 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 different lines from different countries (Section 3.4). This highlights the importance of genotyping the material used when developing mapping populations, specially if the source of the seeds come from different seed banks.

Despite this 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, as expected from 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 couldn't yield to a better map. To improve the map, a cross from the two critical recombinants could be used to repeat a similar analysis, but 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

Talk about Why is R33 diagnostic on the varieties, but maps away?.

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

Figure 3.16: 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.

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 3.16) 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 3.16). 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 the markers developed by breeders show the value of this particular experiment and

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a very
long
quote,
but I'm
finding
hard to
shorten
it.

further confirms that BSA combined with NGS is an effective way to develop novel markers.

Mention other people using a similar strategy since this was published.

Chapter 4

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

4.1 Background.

Describe the list of previously published expression experiments and how they can potentially be used as a framework for new experiments.

Co-expression of homoeologous varies from triplet to triplet (Pfeifer et al., 2014) The silencing is mostly regulated by epigenetic changes as the hybridization events are recent. (Bottley et al., 2006)

Table of experiments to include

The software developed in this section is published in (Borrill et al., 2016).

4.1.1 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 on: aligning the reads to the genome or transcriptome reference and; quantify 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).

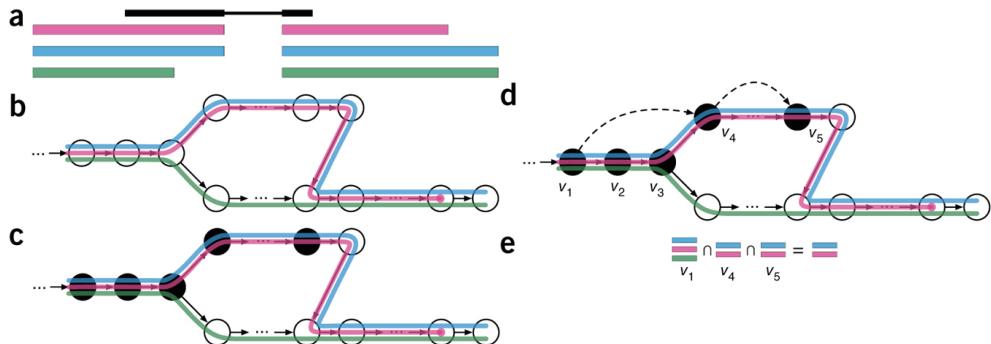


Figure 4.1: "Overview of kallisto. The input consists of a reference transcriptome and reads from an RNA-seq experiment. (a) An example of a read (in black) and three overlapping transcripts with exonic regions as shown. (b) An index is constructed by creating the transcriptome de Bruijn Graph (T-DBG) where nodes (v_1, v_2, v_3, \dots) are k-mers, each transcript corresponds to a colored 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).

For expression analysis only the count of how many reads is required, calculating the best local alignment and the output of each read is unnecessary. **Kallisto** is a tool that generates an index based overlapping k-mers (sequences of size k), which are connected sequentially to represent each transcript (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, an estimate of how many times each transcript appears is estimated (Figure 4.1; Bray et al. 2016).

4.1.2 Relational databases

A Relational databases 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 be-

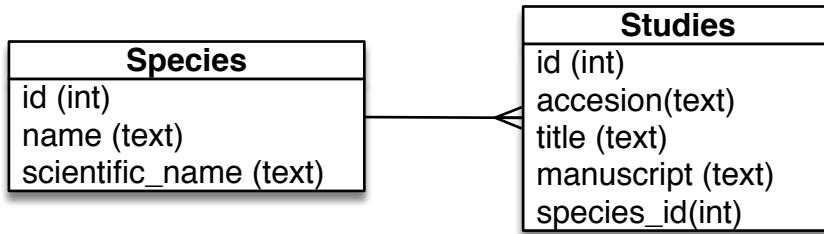


Figure 4.2: Example of a relationship between table. The tables Species and Studies are related. Each study has one species and each species can have several studies.

Table 4.1: 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

tween each other. Continuing with the example, an 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 that concerns to it. A set of columns that have unique values across the table is called a primary key, in our example an extra `id` column is added (Figure 4.2; Codd 1970). The tables 4.1 and 4.2 have the content of their corresponding domains.

4.1.3 SQL

Standard Query Language (SQL) is a common language to retrieve information from relational databases. SQL has operations to select columns and row, join tables, group repeated values and order the results. Those

Table 4.2: 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

simple operations are enough to retrieve the information between tables (Oracle, 2014). The following list shows a brief description of some commands 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 represents all the tables. 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.

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 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 4.3.

Listing 4.1: Join example query

```

1 SELECT
2   species.name,
3   species.scientific_name,
4   studies.accession,
5 FROM species
6 JOIN studies ON species.id = studies.species_id;

```

Table 4.3: 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, represented 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. To ensure that a transaction needs to follow the principles of Atomicity, Consistency, Isolation and Durability (ACID) (Haerder and Reuter, 1983).

Atomicity. All the operation 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 before the transaction started.

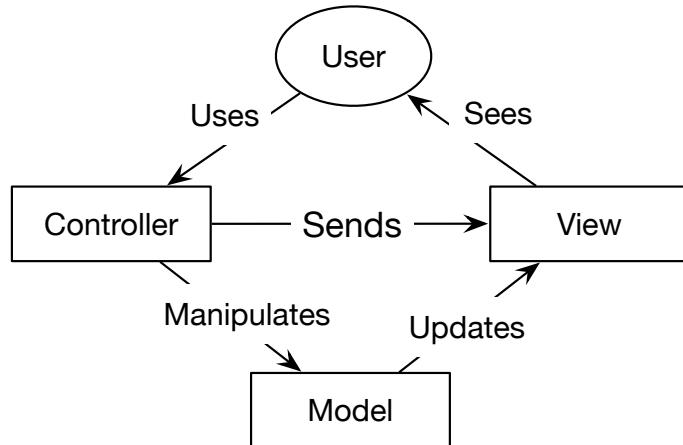


Figure 4.3: MVC interaction between components.

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).

Add explanation of inserts.

4.1.4 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 and modify the models 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 tasks designed to facilitate the 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 the functionality (Rails Guide, 2016).

4.1.5 Data visualisation

In the last couple of decades the amount of information available to any given field has been growing exponentially, in part thanks to the internet. The challenge is to produce tools that help the interpretation of data. 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 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, and not trying to make the system work.

4.1.6 Aims

The aims of expVIP are to:

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

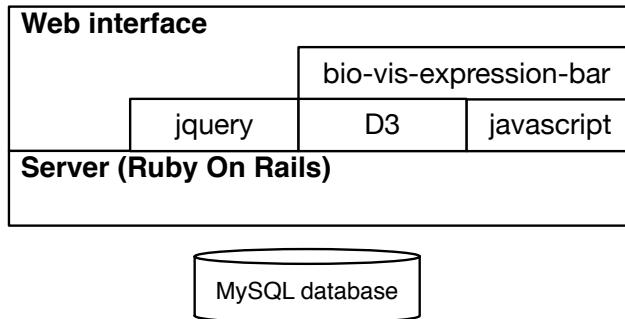


Figure 4.4: General design of expVIP

2. Automate the calculation of the expression values and load them in to the database (Section 4.4).
3. Produce a usable visualisation for said expression values (Section 4.5).
4. Make the system available to the community (Section 4.5).

4.2 General design

One of the main objectives of expVIP is to make the public expression datasets to 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 it (ie Internet Explorer, Chrome). The web server technology used for expVIP is RoR, as it abstracts the MVC metaphor and it is designed to speed the development (Rails Guide, 2016). In order to display the expression data to the users, a BioJS component (Yachdav et al. 2015, Section 4.5). All the data is stored in a MySQL database (Section 4.3) and it is accessed through models developed under RoR (Figure 4.4).

4.3 Database design

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

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

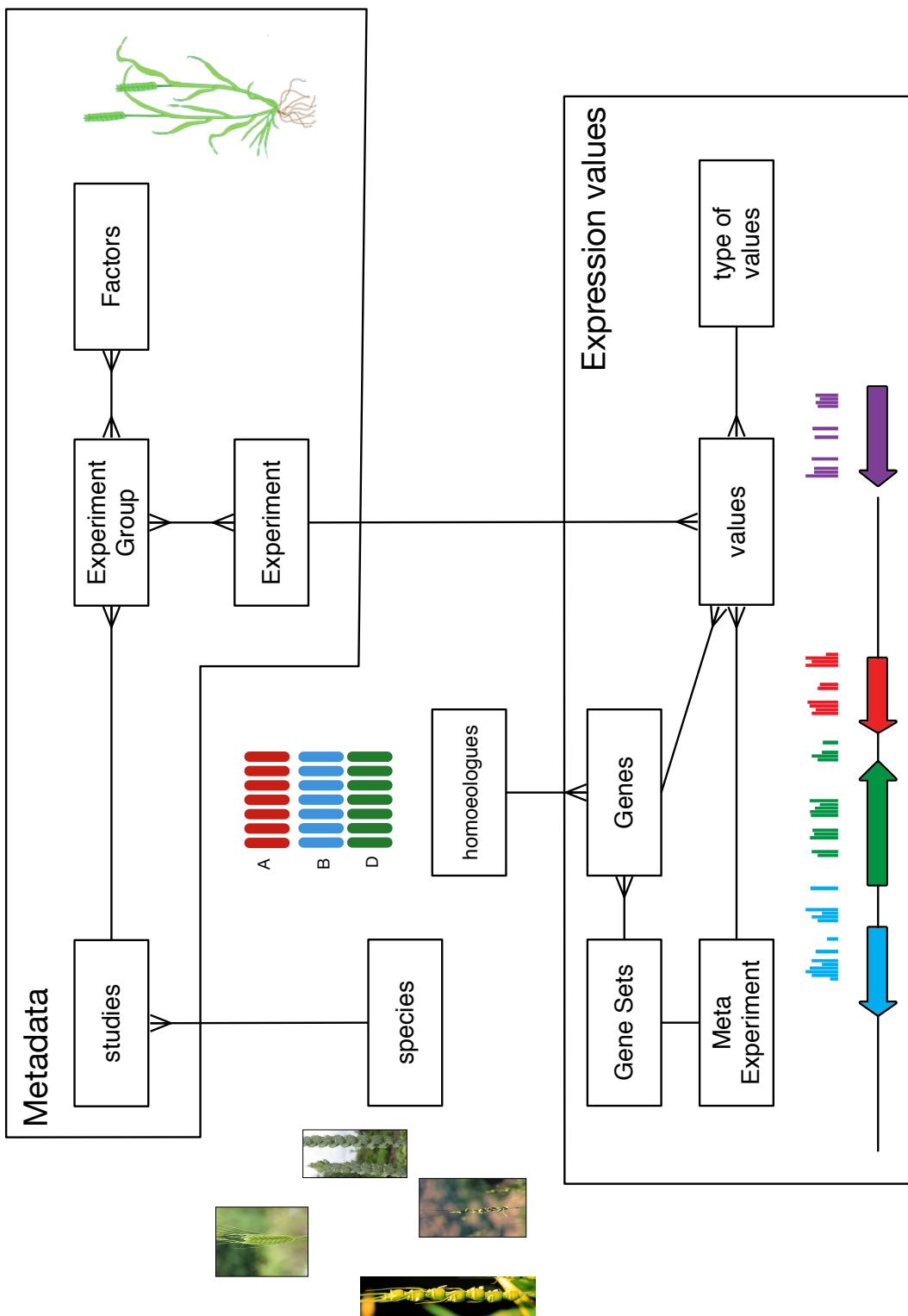


Figure 4.5: Database design. The block on the top stores the meta-data about the experiments and the studies. The bottom block consist on the tables related to the expression values. Species and homoeologues are outside the main blocks as they are not core to the groups. The whiskers in the connections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

Studies Contains the general information of a study, which contain several experiments. The table also contains the reference to the paper where the data is published and the accession 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 doesn't have a fixed number of column 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 expression of each gene and the information for the genes.

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, the use of this table enable the use of several reference gene models on the same database.

Genes are related to a **gene set**, so even if they have the same name coming from different datasets it is possible to distinguish them. This situation is unlikely to occur when using published references, but when joining several *de Novo* gene models.

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

Species contain 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 that a relationship between tables is not unique, such as `experiment_groups` having many `factors` and the `factors` having many `experiment_groups`, storing the relationships is done with an auxiliary table (ie. `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`, the auxiliary tables `ExperimentGroups_Factors` and `experiment_groups_experiments` are used in the query. (Listing 4.2 and Table 4.4).

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

```

1 SELECT
2   experiments.accession,
3   factors.factor,
4   factors.description,
5   experiment_groups.name as expriment_group
6 FROM factors
7 JOIN ExperimentGroups_Factors
8   ON factors.id = ExperimentGroups_Factors.factor_id
9 JOIN experiment_groups
10  ON experiment_groups.id = ExperimentGroups_Factors.
11    experiment_group_id
11 JOIN experiment_groups_experiments
12  ON experiment_groups_experiments.experiment_group_id =
13    experiment_groups.id
13 JOIN experiments
14  ON experiments.id = experiment_groups_experiments.
15    experiment_id
15 WHERE accession = 'DRR003148'
```

Table 4.4: Results of querying the metadata for accession 'DRR003148' (Listing 4.2)

accession	factor	description	expriment 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 4.5

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

```

1 SELECT
2   genes.name as gene,
3   expression_values.value,
4   experiments.accession,
5   type_of_values.name as unit
6 FROM expression_values
7 JOIN genes
8   ON expression_values.gene_id = genes.id
9 JOIN type_of_values
10  ON type_of_values.id = expression_values.
11    type_of_value_id
11 JOIN experiments
12  ON experiments.id = expression_values.experiment_id
13 WHERE
14   genes.name = 'Traes_5BS_0AFC3F795.1'

```

Table 4.5: Results of query to get the values for gene 'Traes_5BS_0AFC3F795.1' (Listing 4.3), only 'Group1' is displayed from the output.

gene	value	accession	experiment	unit
group				
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, the expression values can be calculated with another tool and loaded as

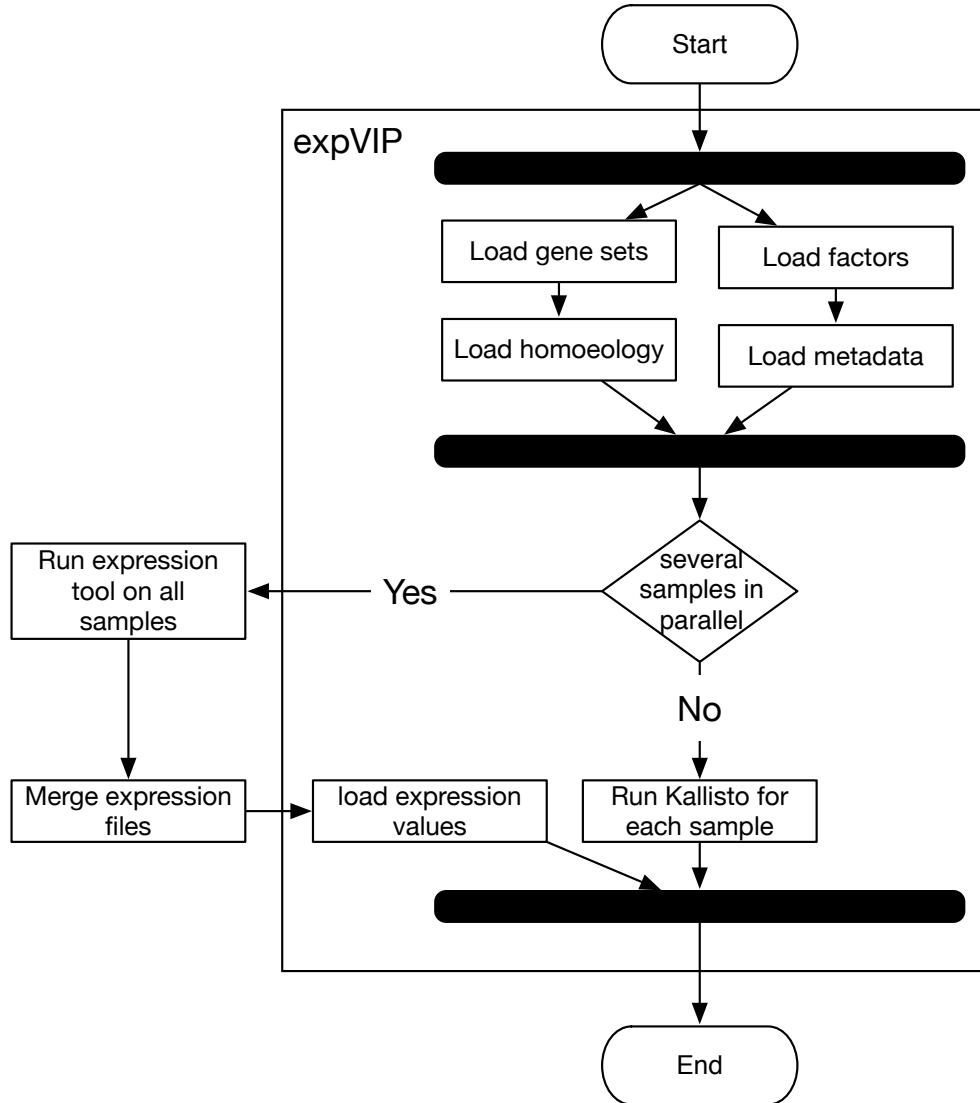


Figure 4.6: The pipeline of loading the data to expVIP. The black lines represent a border of tasks that can be run in parallel or don't require to be done in a particular order.

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 4.6):

factor The category where the factor belongs. In the case of the initial dataset used in expVIP, the grouping factors are: Age,

Table 4.6: 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
...			

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 are sorted chronologically.

name Long description of each factor. This are used as valid values in the metadata.

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 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**. On the absence of this, the only stored value is the name from the >character to the first space (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 TGCTGCTGCTAGGCTTGAAGAGGGTTGCTGGCAAGCTCCAGTCTGCTC
3 GGCAGCTCATTCAAGAGGGCTGTGAGGAGTGCCCCAAGAACGAGGAT
4 GTTGGTCAGGCATGCCGGTTGGCTAGCCCAGATGAGTCAAAGGC
5 AGTAATTGCCAGGGGTGTGAAGGCAATTCCAACTCTGTGAAGCTGT
6 GGCTGCA

```

homoeologues A file containing the homoeologues for the A, B and D genomes. Currently this are the only supported default names. The file also include 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 4.7).

Table 4.7: Example tabular file containing the homoeology across the three genomes.

Gene	A	B	D	Group	Genome
Traes_5BS_0AFC3F795	Traes_5BS_0AFC3F795	Traes_5BS_0AFC3F795	Traes_5DS_0AFC3F795	C204EBAA9	5
Traes_5DS_C204EBAA9	Traes_5DS_C204EBAA9	Traes_5DS_C204EBAA9	Traes_5DS_0AFC3F795	C204EBAA9	5
Traes_7DL_82360D4EE1	Traes_7DL_82360D4EE1	Traes_7DL_82360D4EE1	Traes_7DL_82360D4EE1		7
Traes_2AL_1368BE0AD		Traes_2AL_1368BE0AD	Traes_2AL_1368BE0AD	CD459994C1	2
...					A

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 open with the `csv` library row by row (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, :
4       col_sep => "\t") do |row|
5       factor = Factor.find_or_create_by(:factor=>row["
6         factor"], :description=>row["name"], :name=>row
7         ["short"])
8       factor.order = row["order"].to_i
9       factor.save!
10      end
11    end
12  end
13 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.

Listing 4.6: Task that load genes from a Fasta File

```

1 task :de_novo_genes, [:gene_set,:filename] => :environment
2   do |t, args|
3     ActiveRecord::Base.transaction do
4       gene_set = GeneSet.find_or_create_by(:name=>args[:gene_set])
5       Bio::FlatFile.open(Bio::FastaFormat, args[:filename])
6         do |ff|
7           ff.each do |entry|
8             arr = entry.definition.split(/\s+/)
9             name = arr[0]
10            g = Gene.new
11            g.gene_set = gene_set
12            g.name = name
13            g.cdna = name
14            g.save!
15          end
16        end
17      end
18    end
19  end
20
```

There are two options to load the expression values from the database: A matrix with all the expression values and; run **Kallisto** from expVIP.

The task in Listing 4.7 loads the expression values from a tabular 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 to do the alignments and the reference as a single text.

gene_set. The reference used for the analysisos.

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 trasaction is initiated at the begining 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_experimet`, `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`, which contain the gene name. The ID of the gene 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 reach 1,000, the command to execute 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 make the insertions in batches of 1,000 is to reduce the number of processes running in the database, while keeping the memory usage of the application load. 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.

```

1 task :values, [:meta_experiment, :gene_set, :value_type, :
2   filename ] => :environment do |t, args|
3   ActiveRecord::Base::transaction do
4     conn = ActiveRecord::Base.connection
5     meta_exp = MetaExperiment.find_or_create_by(:name=>args[:meta_experiment])
6     gene_set = GeneSet.find_by(:name=>args[:gene_set])
7     value_type = TypeOfValue.find_or_create_by(:name=>args[:value_type])
8     experiments = Hash.new
9     meta_exp.gene_set = gene_set
10    genes = Hash.new
11    Gene.find_by_sql("SELECT * FROM genes where gene_set_id
12      ='#{gene_set.id}'").each{|g| {genes[g.name] = g.id}}
13    Experiment.find_each{|e| experiments[e.accession] = e.id}
14    count = 0
15    inserts = Array.new
16    CSV.foreach(args[:filename], :headers => true, :col_sep =>
17      "\t") do |row|
18      gene_name = row["target_id"]
19      gene = genes[gene_name]
20      row.delete("target_id")
21      row.to_hash.each_pair do |name, val|
22        val = val.to_f
23        str = "(#{experiments[name]},#{gene},#{meta_exp.id},#{{
24          value_type.id},#{val},NOW(),NOW())"
25        inserts.push str
26      end
27      count += 1
28      if count % 1000 == 0
29        sql = "INSERT INTO expression_values (`experiment_id
30          ,`gene_id`, `meta_experiment_id`, `
31          type_of_value_id`, `value`, `created_at`, `
32          updated_at`) VALUES #{inserts.join(", ")}"
33        conn.execute sql
34        inserts = Array.new
35      end
36    end
37    sql = "INSERT INTO expression_values (`experiment_id`, `
38      gene_id`, `meta_experiment_id`, `type_of_value_id`, `
39      value`, `created_at`, `updated_at`) VALUES #{inserts.
40      join(", ")}"
41    conn.execute sql
42  end
43 end

```

Alternatively, expVIP can execute kallisto on all the samples loaded in the database. For that, a directory with the reads in `fastq`, organized 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 4.4). expVIP takes all the accession for the experiments in the database and search 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 expVIP is executed with its default settings and the results are loaded to 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.

New experiments can be added to the metadata file, or to a new file, and it can be loaded again to update the list of experiments. This allows to keep the database updated as more experiments are 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.

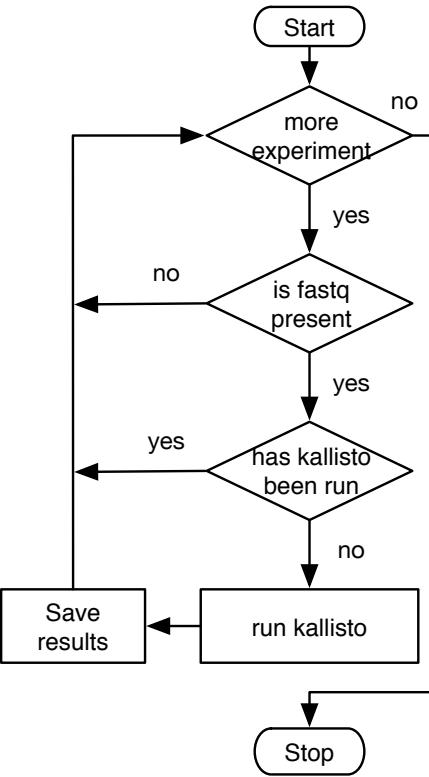


Figure 4.7: Steps to run and load Kallisto

4.5 Graphical interface

With the expression across experiments integrated in a single database, the next challenge is 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 to learn, performance and accuracy were taking in account when designing expVIP. As memorability and satisfaction are subjective, they weren't directly tested 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 of the genes being displayed are loaded from the database in a single transaction. The data is available in the web browser and whenever the user changes anything in the visualisation the 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 bit confusing and the location of the buttons was too far away from the effect they had. 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 interface 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.
3. **Menu options:** has several links on the details of the study and tutorial. The menu can be edited to customize instances of expVIP.
4. **Gene:** shows the currently displayed gene, with a link to Ensembl to the corresponding description.

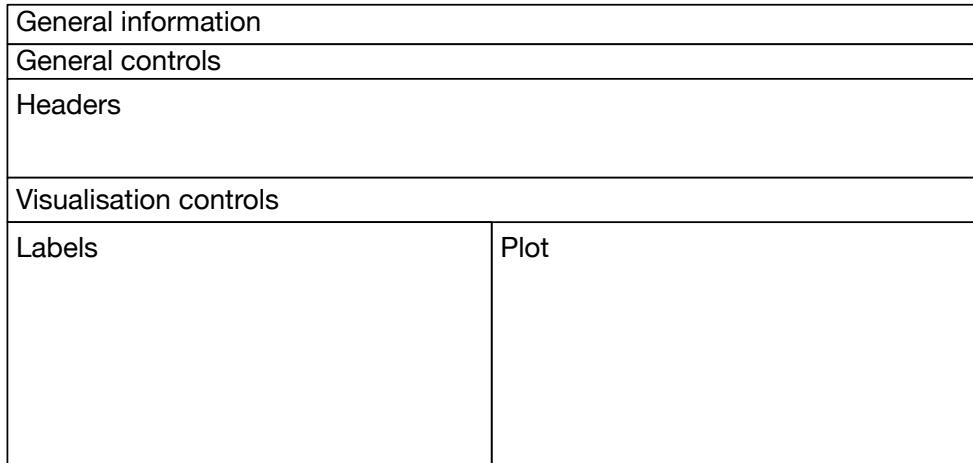


Figure 4.8: expVIP Graphical User Interface components components. The top bar shows a short description of what is displayed. The General and Visualisation controls contain the buttons and menus that enable the interaction with the figure. The Headers, Labels and Plot are the actual components of the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

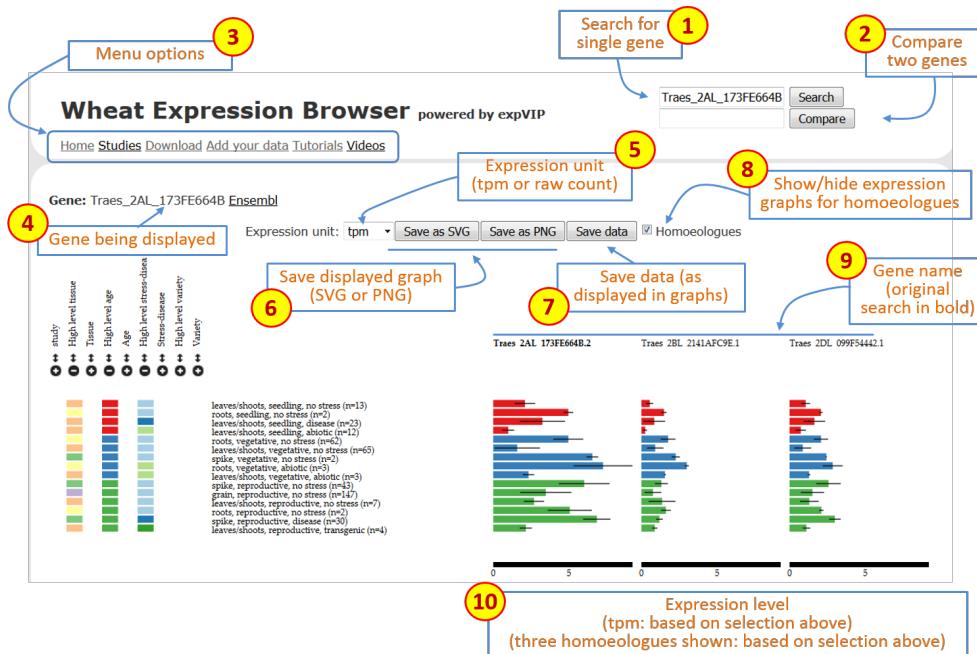


Figure 4.9: Features on expVIP

(a)

Headers	
Labels	Plot

(b)

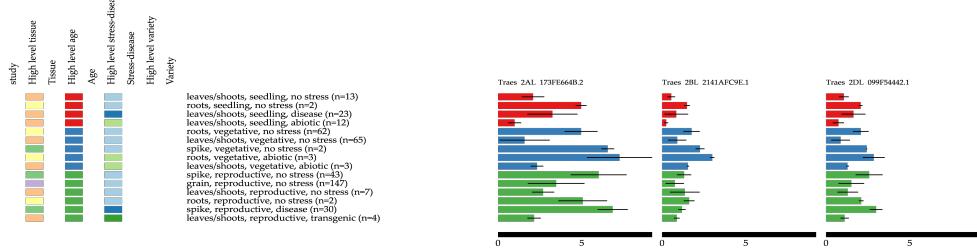


Figure 4.10: expVIP export image. (4.10a) GUI components components exported in image

(4.10b) The exported components from Figure 4.9 .

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 visualization 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.

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

```

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

```

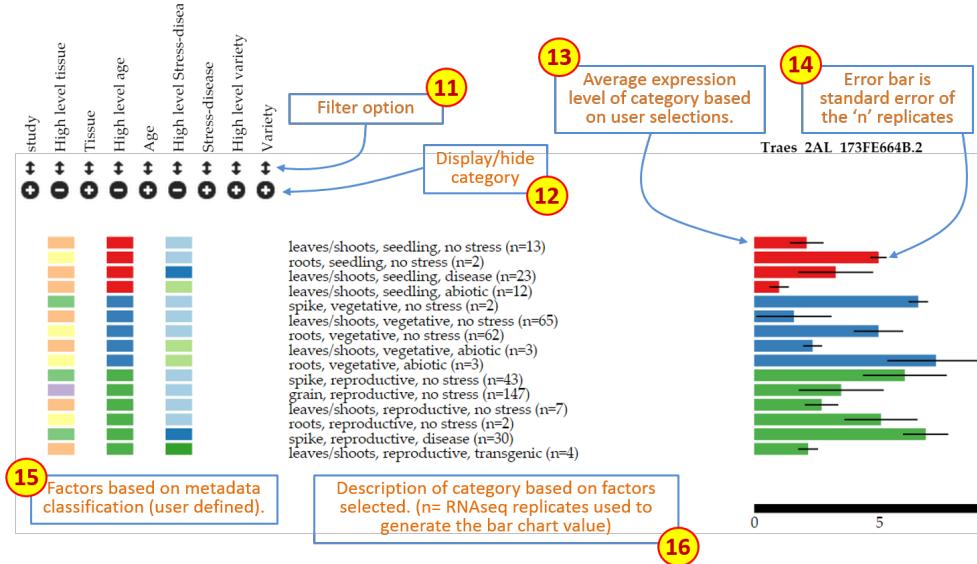


Figure 4.11: Features on expVIP (continued)

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 labeled on this block. If the list of genes 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 re-

moved 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 with 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:** 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 $\log_2(\text{tpm})$ 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 assess 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



Figure 4.12: Features on expVIP for Multiple gene comparisons

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.

4.6 Discussion

The use of previously published studies is a valuable resource. Also, mention that despite the fact that there are several expression/gene browsers, none of them allow comparisons between species and don't consider polyploids.

Things to do: add comparassions between corresponding genes between species

If I have time, I'll add the section about the virtual machine, as I would also need to add something in the background on virtualisation, it can potentially be a sink of time

Chapter 5

General discussion and final remarks

This section wraps up by showing the relationship and importance of a comprehensive approach to data analysis, from the field, genetics, molecular biology and genomics. I will also remark how the technology and the resources have changed in the last 4 years. As at the references used at beginning where superseded during the PhD.

Biology is becoming interdisciplinary

Knowledge from computer science can be applied to produce software for specific needs, but useful for the community

Polypliody has an extra level of complexity (due to homoeologues), but with the current developments of technology is possible to start getting around them.

Future project: PolyInDel.

In the case of wheat, new resources had been coming out year after year, and each one helps to put everything in to a context.

It is more likely to get relevant results in a more effective way using the latest developments.

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	ttagCggattgtcgggatG	ttagCggattgtcggtatA	tgtcCatgaaTcttgtccacG
IWGSC_CSS_4AL_scaff_7106846	Cadenza1772	11277	G	A	hom	hom	tgggatccatgcctacactG	tgggatccatgcctacactA	gatgttGgatttgcgtA
IWGSC_CSS_4AS_scaff_5991335	Cadenza1772	15710	G	A	hom	hom	ctggccctgcgtctcaC	ctggccctgcgtctcaT	gtggaaGttcagaaggaccaG
IWGSC_CSS_4BS_scaff_4956646	Cadenza1772	252	G	A	het*	hom	gcagggttgacttcccgGA	gcagggttgacttcccgGA	tGaggtaGcTaagAAagC
IWGSC_CSS_4DS_scaff_1715962	Cadenza1772	1225	G	A	hom	hom	cagctgtggTatctcaactG	cagctgtggTatctcaactG	CcCtGaaACACcGtttggAT
IWGSC_CSS_5AL_scaff_2763407	Cadenza1772	2119	G	A	hom	hom	gcgacGaaccccgagatctG	gcgacGaaccccgagatctA	gaTggcaAtcgtCgtgcA
IWGSC_CSS_5AS_scaff_1548786	Cadenza1772	12625	C	T	het	het	AtaggcacattgtctgactgA	AtaggcacattgtctgactgA	ggattgggttgtcagcG
IWGSC_CSS_5BL_scaff_10849226	Cadenza1772	2289	C	T	het*	hom	cctgacatcattgttcacgatC	cctgacatcattgttcacgatT	cactccgagggtgtccatgA
IWGSC_CSS_5BS_scaff_2270737	Cadenza1772	2262	G	A	hom	—	attcCTgttgttggCaaatgA	attcCTgttgttggCaaatgA	taaGcacaAAccctccagctG
IWGSC_CSS_1AL_scaff_3022915	Cadenza1661	891	C	T	hom	hom	ccacagtggacttcttggA	ccacagtggacttcttggA	atgtcgttGtcGtgcC
IWGSC_CSS_1AS_scaff_3297240	Cadenza1661	1970	C	T	het	het	cateccccGtttccctC	catcccggatgtttccctC	gctccggatgaaagacG
IWGSC_CSS_1BL_scaff_3828996	Cadenza1661	1340	G	A	hom	hom	acggcgatgttagttAAC	acggcgatgttagttAAC	acgacttggTggctaaC
IWGSC_CSS_1DS_scaff_1884529	Cadenza1661	10575	G	A	hom	hom	aCagatacaAtttcatgcaggC	aCagatacaAtttcatgcaggT	acctgggTTgtccataactTC
IWGSC_CSS_2AL_scaff_6318370	Cadenza1661	19142	C	T	het	—	cgtggcCgaatCtcGacG	cgtggcCgaatCtcGacA	ttcttggtggagccggG
IWGSC_CSS_2AS_scaff_5213460	Cadenza1661	1358	G	A	hom	hom	gtcacgaaCcggctcagG	gtcacgaaCcggctcagA	aggaaaagagggaaaaaGcG
IWGSC_CSS_2BS_scaff_5179331	Cadenza1661	5604	G	A	het	het	actctgtcgaaactgtatacA	actctgtcgaaactgtatacA	gcaGagaatgttcttcaACT
IWGSC_CSS_2DS_scaff_5341235	Cadenza1661	4673	G	A	het	het	ggtagggatctggagatG	ggtagggatctggagatG	gcccggatgtacgatG
IWGSC_CSS_3AL_scaff_4250995	Cadenza1661	7046	G	A	hom	hom	cCaagaaacgggtgttccaG	cCaagaaacgggtgttccaA	ctgcagctgtcccatcatcgT
IWGSC_CSS_3B_scaff_10404421	Cadenza1661	4303	G	A	het	het	ccttcgtcgacCaggacctG	ccttcgtcgacCaggacctA	GCcagactCacAtgcctC
IWGSC_CSS_5DL_scaff_2390496	Cadenza1538	2125	C	T	hom	het	gcagtttatcttcgttgttG	gcagtttatcttcgttgttG	ttctgagaaTgtatgtcGatG
IWGSC_CSS_6AL_scaff_5753680	Cadenza1538	3920	C	T	hom	hom	tgcctccaaattttggaccaaTaaC	tgcctccaaattttggaccaaTaaT	aaatgcaagggttaagttttgT
IWGSC_CSS_6AS_scaff_4425792	Cadenza1538	4307	G	A	hom	het	agatgttgtCggGccA	agatgttgtCggGccA	gctgaagaacacgcgtacaaT
IWGSC_CSS_6BS_scaff_3003630	Cadenza1538	6933	C	T	het	het	ggcgtatgttgtgtcgAG	ggcgtatgttgtgtcgAG	tTgaCttctgggtggcA
IWGSC_CSS_6DL_scaff_3246988	Cadenza1538	9186	G	A	het	het	gctaagaaggatgttggagaaATT	gctaagaaggatgttggagaaATT	aatttctgaagaggttgttatG
IWGSC_CSS_7AL_scaff_4480114	Cadenza1538	3446	C	T	het	—	gataatcccacacggcG	gataatcccacacggcG	tgagccacttgcagttT
IWGSC_CSS_7AS_scaff_4193541	Cadenza1538	8359	C	T	hom	het	agcaatttttgcataattagC	agcaatttttgcataattagT	tcatctGtcttaactctactgtG
IWGSC_CSS_7BL_scaff_6721572	Cadenza1538	9223	C	T	het	het	gctCaggggagaagacaagaaG	gctCaggggagaagacaagaaA	tgctatgaagaattccgacctC
IWGSC_CSS_7BS_scaff_3152545	Cadenza1538	3960	G	A	hom	—	ttagccaaaatcacctgcCgC	ttagccaaaatcacctgcCgT	gCtgcggccatcatcggttaT
IWGSC_CSS_7DS_scaff_3963838	Cadenza1538	2913	G	A	het	het	tCgttgcagaGCTtTtgtG	tCgttgcagaGCTtTtgtG	agaGttATcaagCTactgtcAcA
IWGSC_CSS_1AL_scaff_3903380	Cadenza1469	6193	G	A	hom	hom	ctttcAgagatgaacggG	ctttcAgagatgaacggA	tcGtGagatGtggttGTtA
IWGSC_CSS_1AS_scaff_3287728	Cadenza1469	3817	C	T	het*	hom	ccgaccaAttactaaccG	ccgaccaAttactaaccG	accctttcccAgacatgA
IWGSC_CSS_1BL_scaff_3815304	Cadenza1469	513	G	A	hom	hom	aacatttgccTaCaaaacGC	aacatttgccTaCaaaacGT	acacagcaagtataatgCAAGC
IWGSC_CSS_1DL_scaff_2266648	Cadenza1469	5926	C	T	het	het	caacatgagacacaacacccT	caacatgagacacaacacccT	gtcaacgcgtgaggattG
IWGSC_CSS_1DS_scaff_1906671	Cadenza1469	3697	C	T	hom	hom	tggTGtagacacttggcGA	tggTGtagacacttggcGA	catggcaccaccAcctG
IWGSC_CSS_2AL_scaff_6337088	Cadenza1469	7334	G	A	het*	hom	acaatgccAaggtgacaggTT	acaatgccAaggtgacaggTT	gggagtgttgggtCagaacaT

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	gTgCtcctcGgcacccTT	gTgCtcctcGgcacccTT	gatccGGcaaaactacTG
IWGSC_CSS_2DL_scaff_9832343	Cadenza1469	3262	G	A	het	het	TtgtctaAcagcacCGcagG	TtgtctaAcagcacCGcagA	agatctcggtcagcttTC
IWGSC_CSS_2DS_scaff_5327939	Cadenza1469	3889	G	A	het	het	tttTgccttatgtgactcttagtaC	ttttTgccttatgtgactcttagtaT	gaggccatcacagatagcG
IWGSC_CSS_3B_scaff_10395219	Cadenza1469	1292	G	A	hom	—	agggtcttgctgtctG	agggtcttgctgtctG	ccttctggggcccttataC
IWGSC_CSS_3B_scaff_10592217	Cadenza0580	2994	C	T	het	—	acacgagtatcaagccctC	acacgagtatcaagccctT	tgatacttttgTggCggAG
IWGSC_CSS_3DS_scaff_2596771	Cadenza0580	1037	G	A	het	het	tggttatgCAcaggataatCagG	tggttatgCAcaggataatCagA	tggcaaattgtgatgtcattaggT
IWGSC_CSS_4AL_scaff_7093953	Cadenza0580	9881	C	T	hom	hom	GacaggaaaggccgtaaAC	GacaggaaaggccgtaaAT	ctccAGcaggatggAA
IWGSC_CSS_4BL_scaff_7037448	Cadenza0580	1837	C	T	hom	hom	CgtggaaaaAGtcgaagaacttaAC	CgtggaaaaAGtcgaagaacttaAT	cagttcttcTtCaGagcagataT
IWGSC_CSS_4BS_scaff_4929479	Cadenza0580	10668	G	A	hom	—	tggattttccgcactttC	tggattttccgcactttT	gtaaaacaaggcattcaagatcA
IWGSC_CSS_4DL_scaff_14359838	Cadenza0580	1408	G	A	hom	—	gCtcAttcaggatTGTcTatA	gCtcAttcaggatTGTcTatA	tgaCagaacagtggcatacT
IWGSC_CSS_4DS_scaff_2276484	Cadenza0580	8034	G	A	hom	hom	gccgtgggtatggAgaG	gccgtgggtatggAgaA	cgtccaggattactgatactgcA
IWGSC_CSS_5AL_scaff_2756579	Cadenza0580	5278	G	A	het	het	tgaatggattttcgccccTT	tgaatggattttcgccccTT	ggAAtCCTATgCAGAAgAAAATG
IWGSC_CSS_5BL_scaff_10787208	Cadenza0580	10627	G	A	het	—	gcctctcacatgggagaC	gcctctcacatgggagaT	acgatgtcAggtggGcgT
IWGSC_CSS_5BS_scaff_2282179	Cadenza0580	5267	G	A	het	—	tgtatggctacggctG	tgtatggctacggctG	tcggcccttggaaAtCC
IWGSC_CSS_5DL_scaff_4498073	Cadenza0423	4937	C	T	hom	hom	gcaccctctgtggcatC	gcaccctctgtggcatT	tgagcagaAAGcagccG
IWGSC_CSS_5DS_scaff_2738970	Cadenza0423	2319	C	T	het	—	cgtgagggtgggtatggC	cgtgagggtgggtatggT	tggaaactgttacactgcagtTC
IWGSC_CSS_6AL_scaff_5757109	Cadenza0423	2788	G	A	hom	hom	caggaGcctggcaaataaaAG	caggaGcctggcaaataaaGA	cttcGcgtctttagttcG
IWGSC_CSS_6AS_scaff_4387871	Cadenza0423	2543	G	A	hom	hom	gcatgtaaacaggcggaaaAG	gcatgtaaacaggcggaaaAG	ctcatgtccatgtttaaggT
IWGSC_CSS_6BL_scaff_4271391	Cadenza0423	4660	C	T	hom	hom	tacgtcatgtggtagtgcTA	tacgtcatgtggtagtgcTA	gtttaaggatcatgtggtagtgcA
IWGSC_CSS_6DS_scaff_1880206	Cadenza0423	9159	G	A	het	het	ctgCgaaggctccacaaG	ctgCgaaggctccacaaA	ggatgagaaggttgcattgtcC
IWGSC_CSS_7AS_scaff_4227506	Cadenza0423	952	G	A	het	—	ccatgtttccaatgttagagC	ccatgtttccaatgttagagT	tgcctactgtgtatgcT
IWGSC_CSS_7BL_scaff_6681782	Cadenza0423	1486	C	T	hom	hom	agtaagCGtgacagacaatggG	agtaagCGtgacagacaatggA	AtgtctTtgGtggaaagtacatCA
IWGSC_CSS_7BS_scaff_3160328	Cadenza0423	7801	C	T	het	het	tgttaaatGatacagCtcgcgC	tgttaaatGatacagCtcgcgT	tggaaatggCgttggTTT
IWGSC_CSS_7DS_scaff_407428	Cadenza0423	2051	G	A	het	het	gtcGCgcacatctgcacaG	gtcGCgcacatctgcacaA	actcatAGgtcagccAA
IWGSC_CSS_3AL_scaff_442479	Cadenza0364	3198	C	T	het	het	gagtcTTaagggttgaatggC	gagtcTTaagggttgaatggT	GCaGaTaaCaacaggatcacG
IWGSC_CSS_3AL_scaff_4447942	Cadenza0364	11917	G	A	het	het	gtcataaaaggattgtccctgtgaaG	gtcataaaaggattgtccctgtgaaA	ctcGgatgtggaggaaAG
IWGSC_CSS_3AS_scaff_1557483	Cadenza0364	2547	C	T	het	het	aaagtccatcatgttaccatcaaG	aaagtccatcatgttaccatcaaA	cgaaatccaaacgcctcatA
IWGSC_CSS_3AS_scaff_2648747	Cadenza0364	2688	G	A	het	het	tggAaggcAcaaggggccC	tggAaggcAcaaggggccT	GccgcgtatggagactcG
IWGSC_CSS_3AS_scaff_3304956	Cadenza0364	1017	G	A	het	het	gtccccctgcacacagcttG	gtccccctgcacacagcttA	cctgctggactacaacttcaA
IWGSC_CSS_3AS_scaff_3321091	Cadenza0364	4585	C	T	het	het	caagaatGATgtctgttggA	caagaatGATgtctgttggA	acatgctgaatcgccgaatC
IWGSC_CSS_3AS_scaff_3371333	Cadenza0364	538	G	A	het	het	ggggaaaCgAGcaggcG	ggggaaaCgAGcaggcG	ccgtgccttcacccT
IWGSC_CSS_3AS_scaff_3371815	Cadenza0364	1061	C	T	het	het	atccccacggcacaagAG	atccccacggcacaagAG	aAtggcccttggattcC
IWGSC_CSS_3AS_scaff_3440912	Cadenza0364	4498	G	A	het	het	ccgtaaaactttctgtgtttG	ccgtaaaactttctgtgtttG	atActgacaaaactatcatgttgC
IWGSC_CSS_3B_scaff_10343586	Cadenza0364	2242	G	A	het	—	ggttcTgTcctcttcactG	ggttcTgTcctcttcactA	tgtgttgaaccgcgaagcA
IWGSC_CSS_3AL_scaff_442479	Cadenza0364	3198	C	T	het	het	gagtcTTaagggttgaatggC	gagtcTTaagggttgaatggT	GCaGaTaaCaacaggatcacG
IWGSC_CSS_3AL_scaff_4447942	Cadenza0364	11917	G	A	het	het	gtcafaaaggattgtccctgtgaaG	gtcafaaaggattgtccctgtgaaA	ctcGgatgtggaggaaAG
IWGSC_CSS_3AS_scaff_1557483	Cadenza0364	2547	C	T	het	het	aaagtccatcatgttaccatcaaG	aaagtccatcatgttaccatcaaA	cgaaatccaaacgcctcatA
IWGSC_CSS_3AS_scaff_2648747	Cadenza0364	2688	G	A	het	het	tggAaggcAcaaggggccC	tggAaggcAcaaggggccT	GccgcgtatggagactcG
IWGSC_CSS_3AS_scaff_3304956	Cadenza0364	1017	G	A	het	het	gtccccctgcacacagcttG	gtccccctgcacacagcttA	cctgctggactacaacttcaA
IWGSC_CSS_3AS_scaff_3321091	Cadenza0364	4585	C	T	het	het	caagaatGATgtctgttggA	caagaatGATgtctgttggA	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	ccgtgccttcacccT
IWGSC_CSS_3AS_scaff_3371815	Cadenza0364	1061	C	T	het	het	atccccacggcacagagG	atccccacggcacagagA	aAtggcccttggtgattcC
IWGSC_CSS_3AS_scaff_3440912	Cadenza0364	4498	G	A	het	het	ccgtaaaactttctgtgcctgC	ccgtaaaactttctgtgcctgT	atActgacaaaactacatgtatgc
IWGSC_CSS_3B_scaff_10343586	Cadenza0364	2242	G	A	het	—	ggttcTgTcctcttccactG	ggttcTgTcctcttccactA	tgtgttgaaccgcaga
IWGSC_CSS_5DL_scaff_242342	Cadenza0281	2433	C	T	hom	hom	catggCgacggtGtcctG	catggCgacggtGtcctA	aAccctatTTtggCTACTtCT
IWGSC_CSS_5DL_scaff_4538822	Cadenza0281	1208	G	A	hom	—	acgtcagaacaacccgttgaC	acgtcagaacaacccgttgaT	ttaaatgggtggccac
IWGSC_CSS_6AL_scaff_5813297	Cadenza0281	4532	C	T	hom	—	ggggagggggacgtctcgG	ggggagggggacgtctcgA	ttctctgccaacgattccG
IWGSC_CSS_6AS_scaff_4378990	Cadenza0281	6748	C	T	hom	hom	cccaggttctgtcttttC	cccaggttctgtcttttC	caagtatacggaaatgaaggTgT
IWGSC_CSS_6BL_scaff_4360781	Cadenza0281	5426	C	T	het	het	aCtactcaaattggcttGgtgtAA	aCtactcaaattggcttGgtgtAA	tcaagtccaaatgtCaaagatT
IWGSC_CSS_7AL_scaff_4488310	Cadenza0281	3808	G	A	hom	hom	gttctctttagtagcagccG	gttctctttagtagcagccA	ggcgctttctggcactA
IWGSC_CSS_7BL_scaff_6696509	Cadenza0281	9232	G	A	het	het	gctctaggGgtggcaaAagG	gctctaggGgtggcaaAagA	ggcttGAGtcGcagtG
IWGSC_CSS_7BS_scaff_3143575	Cadenza0281	1866	C	T	het	het	agatgttggagggccgttC	agatgttggagggccgttT	gttggAttgtggcaagtT
IWGSC_CSS_7DL_scaff_3346250	Cadenza0281	1663	G	A	het	het	acgtcagcaacatcccaaC	acgtcagcaacatcccaaT	Tttccaccaggccaa
IWGSC_CSS_7DS_scaff_3933917	Cadenza0281	1243	C	T	het	het	tgCtgaggCttTcacttG	tgCtgaggCttTcacttG	agaggtttttccatGG
IWGSC_CSS_3B_scaff_10626860	Cadenza0148	7847	G	A	het	het	gcagctctgggaggagG	gcagctctgggaggagA	gttaatgtacCTcctagctcG
IWGSC_CSS_3DL_scaff_6915683	Cadenza0148	6904	C	T	het	het	cgtcaaCctgtggcaattG	cgtcaaCctgtggcaattA	tcatgctataatgTcataggT
IWGSC_CSS_4AS_scaff_5929057	Cadenza0148	4238	G	A	hom	hom	gcbcacatcgtagCacctacC	gcbcacatcgtagCacctacT	ttatctgtgaagtgcacaggTCA
IWGSC_CSS_4AS_scaff_5950625	Cadenza0148	10590	C	T	het	het	agaTattCaaaTcggtggAttggC	agaTattCaaaTcggtggAttggT	cctgCtcccccacgtcC
IWGSC_CSS_4AS_scaff_5967119	Cadenza0148	11626	C	T	hom	hom	cgtGacaccccggatG	cgtGacaccccggatG	gacgacgacactgcacgaC
IWGSC_CSS_4DL_scaff_14455742	Cadenza0148	1946	C	T	hom	hom	gCctgaggagatcgcG	gCctgaggagatcgcT	aaccgGtAACTGtGgGcA
IWGSC_CSS_4DS_scaff_2318993	Cadenza0148	4000	C	T	hom	hom	tccaggttggacacatggatggG	tccaggttggacacatggatggA	tgagaTtctgtttccatAttG
IWGSC_CSS_5AL_scaff_2750707	Cadenza0148	4603	G	A	het	het	ccttggtctgtagccatttcaagTaG	ccttggtctgtagccatttcaagTaA	ccaggaTgcAgtcaatattcaaG
IWGSC_CSS_5BL_scaff_10794137	Cadenza0148	9235	C	T	hom	hom	gaagctgttctgcgttG	gaagctgttctgcgttA	agtatcccccataatagcgtG
IWGSC_CSS_5BS_scaff_1646558	Cadenza0148	2916	C	T	het	het	gccGtacactcacatCccttG	gccGtacactcacatCccttA	gcaaTgttccactAtcatccT
IWGSC_CSS_1AL_scaff_3883106	Cadenza0110	27536	C	T	het	het	accttccatactggctG	accttccatactggctGA	gtagaagaacaacatggtaagC
IWGSC_CSS_1BL_scaff_3812829	Cadenza0110	10770	G	A	het*	hom	cccccaactccatcccgG	cccccaactccatcccgA	gGatgttgcgttgcgttgaA
IWGSC_CSS_1DL_scaff_2266648	Cadenza0110	6156	G	A	het	het	actgcgtgttatggacC	actgcgtgttatggacT	ccccatcaactgcacacaAC
IWGSC_CSS_1DS_scaff_1889435	Cadenza0110	8826	C	T	hom	hom	aaccatgaattactcgacagG	aaccatgaattactcgacagA	gccctgtgaagaattgtatcaaacaG
IWGSC_CSS_2AS_scaff_5268634	Cadenza0110	4636	G	A	het	het	gatccatgtgattggcatgtttG	gatccatgtgattggcatgtttA	TgctgtTggatatgcgttacT
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	atttttgtatggctcaatctcgC	atttttgtatggctcaatctcgT	gaacgtTcattctgtacttgcT
IWGSC_CSS_2DS_scaff_5371379	Cadenza0110	2166	C	T	hom	hom	agacacaaaactagtGatgcG	agacacaaaactagtGatgcG	gctgctgagaatgttTgttatttG
IWGSC_CSS_3AL_scaff_4384278	Cadenza0110	1276	C	T	het	het	agcTgaactccccTgtA	agcTgaactccccTgtA	agggacctCgGtggatgaA
IWGSC_CSS_3AS_scaff_3340122	Cadenza0110	1467	C	T	hom	hom	attctAgtgttgcggacatG	attctAgtgttgcggacatA	gagaagactagaaatgttcaGcaT
IWGSC_CSS_5DL_scaff_4554222	Cadenza2103	6528	C	T	het*	hom	gctgcctacaaaagaaaaaaattG	gctgcctacaaaagaaaaaaattA	aTccaaactatCGaTtttgtcataC
IWGSC_CSS_6AL_scaff_5833640	Cadenza2103	7346	C	T	hom	hom	aagaaaaaggccacaaatggttctC	aagaaaaaggccacaaatggttctT	aCTctgTcagtgtttcccaG
IWGSC_CSS_6AS_scaff_4429974	Cadenza2103	3867	G	A	hom	hom	GagatgaAtttattgagcatgtggC	GagatgaAtttattgagcatgtggT	ggttccggcgtcataagT
IWGSC_CSS_6DL_scaff_3307626	Cadenza2103	4970	C	T	hom	hom	tgcagatgttgcgttgcgttA	tgcagatgttgcgttgcgttA	ctaggaagggtgatTTtactGtC
IWGSC_CSS_6DS_scaff_2059604	Cadenza2103	5224	G	A	het	—	gctcaatgcatgcTgagtgG	gctcaatgcatgcTgagtgA	tgtcaatgttattttctgcctG
IWGSC_CSS_7AL_scaff_4552322	Cadenza2103	1412	C	T	het	het	gcaaaaggcTgatactccaacaG	gcaaaaggcTgatactccaacaA	ggcAAGccAgtataaaaagtaaGC

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	—	gcaccttaggatgttagTtatgC	gcaccttaggatgttagTtatgT	gcatgttaggtttttgactgtTA
IWGSC_CSS_7DL_scaff_3382467	Cadenza2103	3473	C	T	hom	—	GGTtctgCaGTTCATAAActcatC	GGTtctgCaGTTCATAAActcatT	attgaatacaactgatacGaaGactC
IWGSC_CSS_3B_scaff_10457010	Cadenza0277	10599	G	A	het	het	aaccttggcccgacaacaC	aaccttggcccgacaacaT	actggctgcacgagaggG
IWGSC_CSS_3B_scaff_10593852	Cadenza0277	10124	C	T	het	het	tgacaggggacgtatacaG	tgacaggggacgtatacaA	gtctcaaCTtACAttAccatcagC
IWGSC_CSS_3DS_scaff_2583390	Cadenza0277	663	G	A	hom	hom	actgcactatacatActtCtgC	actgcactatacatActtCtgT	tcCacctggacagcaagtG
IWGSC_CSS_4AL_scaff_7093953	Cadenza0277	10004	C	T	hom	hom	ccttgtatccaatggATtgTtttgG	ccttgtatccaatggATtgTtttgA	tcccacaataaaaggaaagagC
IWGSC_CSS_4AL_scaff_7176064	Cadenza0277	6220	C	T	het	het	gtgcgtgtTCgcctG	gtgcgtgtTCgcctG	atgttcggggatgggG
IWGSC_CSS_4DL_scaff_14122349	Cadenza0277	1010	C	T	hom	hom	gtgcgtgtCttgtGA	gtgcgtgtCttgtGA	ggaacaggccaaaggagG
IWGSC_CSS_5AL_scaff_2736916	Cadenza0277	4296	G	A	het	het	aagaactATgAaaGtaacacacgaC	aagaactATgAaaGtaacacacgaT	ttcGTTtTaAgGcAttCtcG
IWGSC_CSS_5BL_scaff_10883744	Cadenza0277	2080	C	T	hom	hom	gcctcttTgttTagectcaG	gcctcttTgttTagectcaA	cgacaagggtcgatTgcA
IWGSC_CSS_1AL_scaff_3932013	Cadenza0548	11765	C	T	hom	hom	accgccaaCccaagacaG	accgceaaCccaagacaA	ccattaaGccgTgcAacG
IWGSC_CSS_1BS_scaff_3417505	Cadenza0548	373	C	T	het	het	gtgtgtgggAGgtgGaG	gtgtgtgggAGgtgGaA	tggtcGccagttgttgA
IWGSC_CSS_2AS_scaff_5305619	Cadenza0548	2786	C	T	hom	hom	atacagatgcctAAgtggTtC	atacagatgcctAAgtggTtT	ggaagacaAtGtcaggtaC
IWGSC_CSS_2AS_scaff_5306489	Cadenza0548	46953	T	G	het	wt	aggttgcattgtccatagaaGT	aggttgcattgtccatagaaGG	aggctaTAactctgtACAgT
IWGSC_CSS_2BL_scaff_7984123	Cadenza0548	11660	G	A	het	het	catttgtggcatagtaatcgtacaG	catttgtggcatagtaatcgtacaA	aatacattgaggaaatcaaagccC
IWGSC_CSS_2DL_scaff_9907477	Cadenza0548	1363	C	T	hom	hom	tgccctcccttgcagaaC	tgccctcccttgcagaaT	ggcaaaacctgttgccatC
IWGSC_CSS_2DS_scaff_5330886	Cadenza0548	5449	G	A	hom	hom	gcatgtccatttatactgaaCgtG	gcatgtccatttatactgaaCgtA	catgtcttcttcgtgacC
IWGSC_CSS_3AL_scaff_4449951	Cadenza0548	633	C	T	het	het	tccaaacctaacagtctaactaG	tccaaacctaacagtctaactaA	gtctgcagTGCaatgtgC
IWGSC_CSS_3B_scaff_10479889	Cadenza0097	3339	C	T	hom	—	ttgTttctGgagaagatgcCG	ttgTttctGgagaagatgcA	ggtgcattcaGggcA
IWGSC_CSS_3B_scaff_10562262	Cadenza0097	7819	C	T	het	het	agaggggtgtctatccatAttgG	agaggggtgtctatccatAttgA	aggcgttccaaaggcttc
IWGSC_CSS_4AL_scaff_7040796	Cadenza0097	10772	G	A	hom	hom	acacaacattgcaccagaG	acacaacattgcaccagaA	CAatCgtattgttcTtc
IWGSC_CSS_4AL_scaff_7063488	Cadenza0097	6360	C	T	het	het	gcctctcacCttAatttgaagctgC	gcctctcacCttAatttgaagctgT	aggcagtgggatgtgaaggTT
IWGSC_CSS_4AL_scaff_7091701	Cadenza0097	5050	G	A	het	het	catgagcatctggggagaaatG	catgagcatctggggagaaatA	agcaaggaaAtaatgaacggaaA
IWGSC_CSS_4DS_scaff_1845841	Cadenza0097	7110	G	A	hom	hom	aatgTAgctccccatCgG	aatgTAgctccccatCgA	actgaaacTgcatacgTtatggA
IWGSC_CSS_5AL_scaff_2767581	Cadenza0097	3737	G	A	het	het	gagagggtctcaactAtcgC	gagagggtctcaactAtcgT	cgTcatcacaatattgtcgG
IWGSC_CSS_5BL_scaff_10784643	Cadenza0097	1568	C	T	hom	hom	agaaaTAcatggatggatggA	agaaaTAcatggatggatggA	catctCCttccaCgGaaaG
IWGSC_CSS_1AL_scaff_3952258	Cadenza2092	8107	C	T	het	—	tgtagtagaaaaattgcacatgtG	tgtagtagaaaaattgcacatgtG	tgccacattgcacatgtG
IWGSC_CSS_1BL_scaff_3858008	Cadenza2092	10278	G	A	hom	hom	tttgagcaggcaggatcgC	tttgagcaggcaggatcgT	actcacggcttatacActattC
IWGSC_CSS_1DL_scaff_2265172	Cadenza2092	9094	C	T	hom	hom	tgcaTGTcattttgtttatcgC	tgcaTGTcattttgtttatcgT	agtgtccaaactccGttcatC
IWGSC_CSS_2AL_scaff_6435867	Cadenza2092	16201	G	A	hom	hom	tttctgTacccttaacgtcaattgaC	tttctgTacccttaacgtcaattgaT	gtgaggatgtgaggtaagacC
IWGSC_CSS_2AL_scaff_6439430	Cadenza2092	25101	C	T	het	—	caagaaaggCagCtCagC	caagaaaggCagCtCagT	tcGttAcTtttcActggtaA
IWGSC_CSS_2DL_scaff_9760848	Cadenza2092	4733	C	T	het	het	gcaccatgggtctcggtA	gcaccatgggtctcggtA	tcagtcatGCTCtgTCTG
IWGSC_CSS_3AL_scaff_4407012	Cadenza2092	2785	C	T	hom	hom	acatatAgttttctatccacatC	acatatAgttttctatccacatT	acctcttcatgttaataggttgT
IWGSC_CSS_3AS_scaff_3441108	Cadenza2092	541	G	A	het	het	GtgatgaccttgcggG	GtgatgaccttgcggG	aggcaTgacaaCgcgcaA
IWGSC_CSS_3B_scaff_10449827	Cadenza1551	4779	G	A	hom	hom	ggcaaggtaagaaacGgtC	ggcaaggtaagaaacGgtT	aCagaGtgggttagaggcaG
IWGSC_CSS_3B_scaff_10550638	Cadenza1551	3250	C	T	het	het	ctccctcacatgttgcggC	ctccctcacatgttgcggT	gcaacAtTttgataactgcaagG
IWGSC_CSS_3DL_scaff_6945816	Cadenza1551	589	C	T	hom	hom	agcatctacccgtcaaCaataC	agcatctacccgtcaaCaataT	TgtgccCTctgaAtatTTcaTG
IWGSC_CSS_3DL_scaff_6954177	Cadenza1551	3508	C	T	het	het	tgttagcatcacatTAacttctG	tgttagcatcacatTAacttctG	gcttggataaacCttacgacA
IWGSC_CSS_4AS_scaff_5938272	Cadenza1551	19080	G	A	hom	hom	agAcCccgAtgcctatG	agAcCccgAtgcctatG	GggAgatAcaggtaaaActcTtcG
IWGSC_CSS_4AS_scaff_5977594	Cadenza1551	11092	C	T	het	het	gccttgattcgaaacaacaaaC	gccttgattcgaaacaacaaaT	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	cggtgatattTtttagacttcgacgC	cggtgatattTtttagacttcgacgT	ggcagttcagcGaccatT
IWGSC_CSS_5BL_scaff_10889480	Cadenza1551	2530	G	A	hom	hom	gagcttaactcgccagatggaG	gagcttaactcgccagatggaA	tccatgCAacGccttgT
IWGSC_CSS_3B_scaff_10528396	Cadenza2088	8059	G	A	hom	—	ctttccgtccgtaaagcaataG	ctttccgtccgtaaagcaataA	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	ccaGaggctgtTGcatTTT
IWGSC_CSS_4AL_scaff_7126302	Cadenza2088	3627	G	A	hom	hom	gttccaaaacaaggatggctAatttgC	gttccaaaacaaggatggctAatttgT	cacaaggatatgaagCTttctagA
IWGSC_CSS_4BL_scaff_7041808	Cadenza2088	10234	G	A	hom	hom	tcaatggatgagggtgtttC	tcaatggatgagggtgtttT	ccatagcagcatcagccacA
IWGSC_CSS_5AL_scaff_2794167	Cadenza2088	13162	G	A	het	—	agtattcaggacaaggcatCttCaG	agtattcaggacaaggcatCttCaA	caatgaaacctctcgaaagaGaG
IWGSC_CSS_5BL_scaff_10889232	Cadenza2088	3885	G	A	het	het	cTcaaccacaatggcaAatC	cTcaaccacaatggcaAatT	tccttcataatcatcaatgttgG
IWGSC_CSS_5BS_scaff_2267405	Cadenza2088	11113	C	T	hom	hom	ctttgatgtccatggctctTG	ctttgatgtccatggctctTA	tgatttggTCgtttAgatttGA
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	gttcctagtcgtccatcaG	gttcctagtcgtccatcaA	cttcacacCCGagataatGtattG
IWGSC_CSS_4AL_scaff_7153568	Cadenza1409	13073	C	T	hom	hom	tccgaccGAtcaaccttgG	tccgaccGAtcaaccttgA	gaccggaaacttcggcC
IWGSC_CSS_4DL_scaff_14314966	Cadenza1409	2010	G	A	het	hom	gttggccccctctCAGgG	gttggccccctctCAGgA	cggcgTcacaAgttgCcT
IWGSC_CSS_4DS_scaff_2324074	Cadenza1409	7606	G	A	het	het	tGcatgaaaatgttGcaGaG	tGcatgaaaatgttGcaGaA	gggtaAgttcaAAactGaaGtgaG
IWGSC_CSS_5AS_scaff_1517889	Cadenza1409	3561	G	A	het	het	tctcgacatctcccggttaC	tctcgacatctcccggttaT	gtgcctggAACATTGCTTATT
IWGSC_CSS_5AS_scaff_1523866	Cadenza1409	8054	G	A	hom	—	ggtgatctaccgccaGgaC	ggtgatctaccgccaGgaT	tcctgcagCcTetcctcA
IWGSC_CSS_5BL_scaff_10917655	Cadenza1409	19073	G	A	hom	hom	caaatacgatcggaaaaggatggC	caaatacgatcggaaaaggatggT	cgcttcatactacaAaatatgtcT
IWGSC_CSS_1AL_scaff_3886649	Cadenza1599	5204	C	T	het	het	tgtatcccaaccacatGcC	tgtatcccaaccacatGcT	ggactgactgcgaccatatttaG
IWGSC_CSS_1BL_scaff_3810267	Cadenza1599	6634	C	T	hom	hom	ccCaggaaatggacaccC	ccCaggaaatggacaccT	cgcaggcgaagatgtgaTtG
IWGSC_CSS_1DL_scaff_2291677	Cadenza1599	12856	C	T	hom	hom	GgttagacaatgcgcgaG	GgttagacaatgcgcgaA	cctctcttcacacGCcG
IWGSC_CSS_2AL_scaff_6354492	Cadenza1599	7566	G	A	het	het	gGagaatgcACAgAAcTtctgG	gGagaatgcACAgAAcTtctgA	ttccgaagaaccacaTccTG
IWGSC_CSS_2AS_scaff_5282937	Cadenza1599	9736	G	A	het	het	gctgttagattttatagctgtatG	gctgttagattttatagctgtatG	cacCagaattttCactgattTC
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IWGSC_CSS_2DL_scaff_9897981	Cadenza1599	5627	C	T	het	het	cttggtgtCTgattgttactC	cttggtgtCTgattgttactT	gTttgtCTctctgtactTtgtG
IWGSC_CSS_3AL_scaff_4446105	Cadenza1599	1765	G	A	hom	—	aaatgtttctaCcgctagtG	aaatgtttctaCcgctagtA	tttAgaggcaatagctTatatgcT

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	ccacacccTggcctcgC	ccacacccTggcctcgT	gtgatttgcacggggAG
IWGSC_CSS_1BL_scaff_3897513	Kronos3085	1515	C	T	Het	Het	gttccactCGgttcTcgC	gttccactCGgttcTcgT	acAaggactgttcagaGaC
IWGSC_CSS_2AL_scaff_6434745	Kronos3085	3424	C	T	Het	Het	cctcGgtttgcaatttctatgC	cctcGgtttgcaatttctatgT	gGCaaTggcataacaacagataA
IWGSC_CSS_3AS_scaff_3408995	Kronos3085	732	C	T	Het	Het	aggccatttgcattccgC	aggccatttgcattccgT	ggTgttaTccagAaccTgAG
IWGSC_CSS_3B_scaff_10708748	Kronos3085	2675	G	A	Het	Het	gttgcattgcgttacccacG	gttgcattgcgttacccacA	gtaacaatctgatgttgcgtacC
IWGSC_CSS_4AL_scaff_7132733	Kronos3085	1799	C	T	Hom	Hom	caccctgtggatgcacccT	caccctgtggatgcacccT	aCcGcctaGaaagaagactTC

IWGSC contig	Line	Pos	WT	Mut	Predicted	<i>M</i> ₄	Primer 1 (Kronos)	Primer 2 (mutant)	Common Primer
IWGSC_CSS_5AS_scaff_1534693	Kronos3085	4605	C	T	Het	Het	cagttctggccctcAtC	cagttctggccctcAtT	gtACtccacgAgtcaTgAG
IWGSC_CSS_6AS_scaff_4361911	Kronos3085	8857	G	A	Het	Het	tcacgaaagacgacttcaacctcC	tcacgaaagacgacttcaacctcT	catgagggtgcgtcatctccatA
IWGSC_CSS_6BS_scaff_3008326	Kronos3085	1528	G	A	Het	Het	ccatgttgtactgggttgC	ccatgttgtactgggttgT	ggaagcatggCaatgtcA
IWGSC_CSS_7AS_scaff_4214385	Kronos3085	27835	C	T	Hom	Hom	cgtacccctggggaaG	cgtacccctggggaaG	cttggcgtacgtgtataagacT
IWGSC_CSS_1AL_scaff_3929964	Kronos3191	1336	C	T	Het	Het	tttcggccataacctgacatC	tttcggccataacctgacatT	attgcctccaggcttgcAG
IWGSC_CSS_1BL_scaff_3899789	Kronos3191	7925	C	T	Het	Het	acttcacTggcagcagC	acttcacTggcagcagT	caaagtgtgtcccatGtA
IWGSC_CSS_2AL_scaff_6426728	Kronos3191	1481	G	A	Hom	Hom	gaaActgccgcgtCgC	gaaActgccgcgtCgT	ccaGcaGcgtgtgagaaA
IWGSC_CSS_2BL_scaff_7960273	Kronos3191	690	C	T	Hom	Hom	gcccattatccttaggcG	gcccattatccttaggcT	acatgcattgtgtgactG
IWGSC_CSS_3AS_scaff_3286603	Kronos3191	2975	G	A	Het*	Hom	ccgttgtgggttgtgtggG	ccgttgtgggttgtgtggA	gaaggAACtgcTcaTgcAG
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IWGSC_CSS_7BL_scaff_6739382	Kronos3191	12261	G	A	Hom	Hom	gagacaaggatgttgaattgtc	gagacaaggatgttgaattgtc	CggagtacTcatttcccG
IWGSC_CSS_1AS_scaff_3276389	Kronos3288	9720	C	T	Hom	Hom	aCcaGcaggaccAatgtctC	aCcaGcaggaccAatgtctT	atgatgcacccatgcCA
IWGSC_CSS_2AL_scaff_6367515	Kronos3288	6976	G	A	Het	Het	caggtcgagTgtctccG	caggtcgagTgtctccG	gggggtatCtggaggG
IWGSC_CSS_2AL_scaff_6422019	Kronos3288	4523	G	A	Het	Het	cgctaggccctgcatacG	cgctaggccctgcatacA	acgcAcgtcaaggcgtA
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IWGSC_CSS_4AS_scaff_5962359	Kronos3288	13049	G	A	Het	Hom	ccatcaagaaggatcagggtcA	ccatcaagaaggatcagggtcA	accatgcggccatgtcA
IWGSC_CSS_6AL_scaff_5777873	Kronos3288	6853	G	A	Het	Het	gagtgcaccccttcgttT	gagtgcaccccttcgttT	ggagaacacgtactcgG
IWGSC_CSS_6AS_scaff_4392100	Kronos3288	3434	C	T	Het	Het	atggaaagcagggtgaccG	atggaaagcagggtgaccA	ggAaggcggaaatggaaacAA
IWGSC_CSS_7BL_scaff_6744240	Kronos3288	9772	G	A	Het	Het	agctgtctctctacttcaaG	agctgtctctctacttcaaA	caggctgttcttgagctc
IWGSC_CSS_1AL_scaff_3887185	Kronos3413	9708	C	T	Hom	Hom	gcacgccttattcgaggtaaA	gcacgccttattcgaggtaaA	AgaaacagcagcgcA
IWGSC_CSS_2BS_scaff_3381362	Kronos3413	5160	C	T	Het*	Hom	caacttgcgtgttgtG	caacttgcgtgttgtA	tgAgaattctgacGaaaaAG
IWGSC_CSS_3AS_scaff_3296605	Kronos3413	6154	G	A	Het	Het	ctggtaacgggtctagC	ctggtaacgggtctagT	cacgactgagacatggGA
IWGSC_CSS_3B_scaff_10693516	Kronos3413	12632	C	T	Het	Het	ctaggcttggacaaaaggC	ctaggcttggacaaaaggT	agcttgcattatctggcatT
IWGSC_CSS_5AS_scaff_1547699	Kronos3413	2686	G	A	Het	Het	gtCtacaccccttccaaatcg	gtCtacaccccttccaaatcg	gacggctttaatgtgtc
IWGSC_CSS_5BL_scaff_10856077	Kronos3413	5853	G	A	Het	Het	agagcttccccatgtc	agagcttccccatgtc	acgCacatttAatagctgaaG
IWGSC_CSS_6AL_scaff_5750718	Kronos3413	11046	G	A	Hom	Hom	cacgcTtcccgacttataG	cacgcTtcccgacttataA	AgacgatgtatcgaggattcA
IWGSC_CSS_7AL_scaff_4433177	Kronos3413	3511	C	T	Het	Het	GaTgtccGtcaggctgG	GaTgtccGtcaggctgA	cactactggacaaagcttG
IWGSC_CSS_7BL_scaff_6742567	Kronos3413	667	C	T	Het	Het	gttgcgttgcgtggcagaC	gttgcgttgcgtggcagaT	cattttgcacccgtgtcTG
IWGSC_CSS_1AL_scaff_3976389	Kronos3935	10941	C	T	Hom	Hom	ggtgaggagatcggCgtA	ggtgaggagatcggCgtA	cagtcattatcatgaggggtcA
IWGSC_CSS_1BL_scaff_3873362	Kronos3935	1392	G	A	Het	Het	cagatctgaaggctaGacatG	cagatctgaaggctaGacatA	actaccagaatcgcacaaaaAC
IWGSC_CSS_2BL_scaff_7882382	Kronos3935	2721	C	T	Het	Het	gcaagctaaggatgtacccgt	gcaagctaaggatgtacccgt	gccacagttaggagaaagactT
IWGSC_CSS_3AL_scaff_4242376	Kronos3935	2410	C	T	Het	Het	agaacccaaaacccgTacttaG	agaacccaaaacccgTacttaA	gtagGgtCcattCtaaagcttG
IWGSC_CSS_3B_scaff_10485067	Kronos3935	3349	C	T	Hom	Hom	gttgcggcaacttcaactG	gttgcggcaacttcaactA	gcaatttcccttaTccgcagT
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IWGSC_CSS_4BL_scaff_7019402	Kronos3935	9081	C	T	Het	Het	tgcaatcatgtgtgagctG	tgcaatcatgtgtgagctG	agcatgatcccttagaaCatac
IWGSC_CSS_5BL_scaff_10842786	Kronos3935	3304	G	A	Het	Het	tggttcccGaaggctgaaC	tggttcccGaaggctgaaT	cgcatacttgcacaaTgagcAC
IWGSC_CSS_6BS_scaff_3045205	Kronos3935	2293	G	A	Het	Het	aaggaccaaggccaaactctcG	aaggaccaaggccaaactctcA	agtgtaccaaggccaaatgtcG

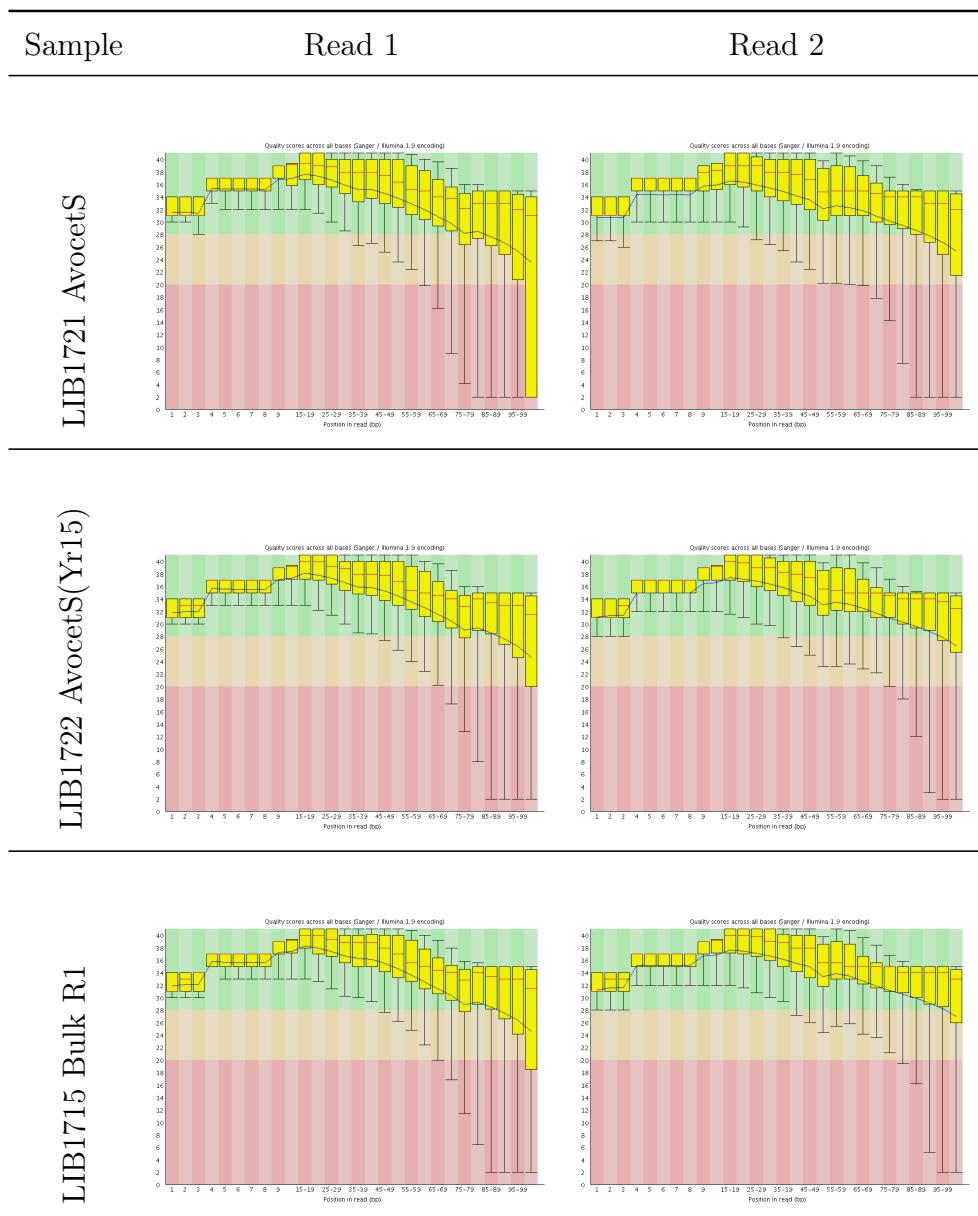
APPENDIX A. SUPPLEMENTAL TABLES

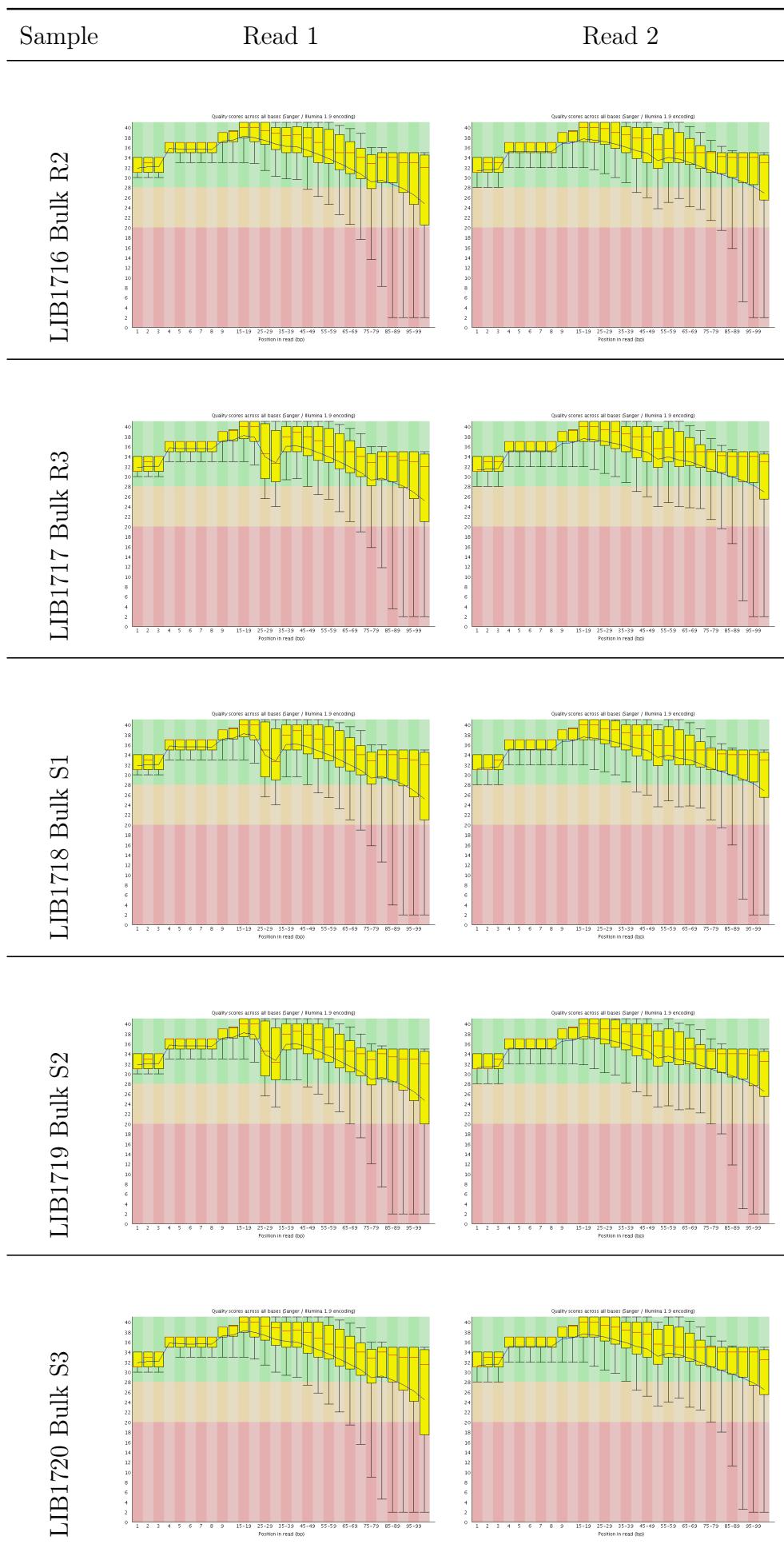
IWGSC contig	Line	Pos	WT	Mut	Predicted	M_4	Primer 1 (Kronos)	Primer 2 (mutant)	Common Primer
IWGSC_CSS_7AL_scaff_4555249	Kronos3935	4487	C	T	Het	Het	cAgtgctcgagatggcgC	cAgtgctcgagatggcgT	cCttgcacccctcgatT
IWGSC_CSS_1BL_scaff_3918498	Kronos4240	6096	G	A	Het	Het	ttgcattccccaaagaagaG	ttgcattccccaaagaagaA	tggcgaaactgtaatgtG
IWGSC_CSS_2BS_scaff_5131713	Kronos4240	5900	G	A	Het	Het	cctttatcgaggaaagagacacC	cctttatcgaggaaagagacacT	caccattgttaggttcTTtC
IWGSC_CSS_5AL_scaff_2769540	Kronos4240	9626	C	T	Het	Het	tgCagtgtggaaacggaG	tgCagtgtggaaacggaA	catgagtGagatcttcgtC
IWGSC_CSS_5BL_scaff_10871091	Kronos4240	7062	G	A	Het	Het	gccaaggAaccataacctgC	gccaaggAaccataacctgT	GgacttggcAaccggA
IWGSC_CSS_6AL_scaff_5800333	Kronos4240	2360	G	A	Het	Het	cgacaggattgtgagCgC	cgacaggattgtgagCgT	tcaagatgtcaagattcatcT
IWGSC_CSS_7BL_scaff_6716931	Kronos4240	2613	G	A	Het	Het	gGtgGgtattTgcttggtaG	gGtgGgtattTgcttggtaA	tgGtgactgcacaGtGtA
IWGSC_CSS_2BL_scaff_8029221	Kronos4346	2860	G	A	Het	Het	tgcttccgtctgtcT	tgcttccgtctgtcT	atTtgcattCgAtcggcC
IWGSC_CSS_3B_scaff_10460714	Kronos4346	14359	C	T	Hom	Hom	ctacccatcgacatG	ctacccatcgacatA	agcacccacttggacG
IWGSC_CSS_4AS_scaff_5989735	Kronos4346	6404	G	A	Hom	Hom	acgcattctaaccatcagcC	acgcattctaaccatcagcT	actcaagataccaCcgacG
IWGSC_CSS_5BL_scaff_7648030	Kronos4346	6893	C	T	Het	Het	tacccttctactggcagG	tacccttctactggcagA	ttttcagggAACAGGTtAC
IWGSC_CSS_6AL_scaff_5755840	Kronos4346	778	C	T	Het	Het	atcgagtaactgtcacCgC	atcgagtaactgtcacCgT	acctgcattgtcaCatccC
IWGSC_CSS_6BS_scaff_2972151	Kronos4346	7876	G	A	Hom	Hom	gcagaatgtcActgtttG	gcagaatgtcActgtttA	gcttggactggcatttATG
IWGSC_CSS_7AL_scaff_4542983	Kronos4346	18700	G	A	Het	Het	gcaggcgtAccggatacT	gcaggcgtAccggatacT	catctgccGgttaaacatcG
IWGSC_CSS_7BS_scaff_3098098	Kronos4346	5183	C	T	Het	Het	gCgatatggtaacttgcataG	gCgatatggtaacttgcataA	ttacattgttataGTtgtCcgG
IWGSC_CSS_1AS_scaff_3259804	Kronos4485	219	C	T	Het	Het	gtcggcacaaccccttgC	gtcggcacaaccccttgT	gcttcttaaggaggcGA
IWGSC_CSS_2AL_scaff_6315418	Kronos4485	10490	G	A	Hom	Hom	gcccctctaaCttctcagC	gcccctctaaCttctcagT	ttcagacgtCgaggatttCC
IWGSC_CSS_2BS_scaff_5181092	Kronos4485	3742	G	A	Het	Het	TggccagcacactgcAG	TggccagcacactgcA	tggacgttagTgtatggAAaT
IWGSC_CSS_3B_scaff_10425015	Kronos4485	2372	C	T	Het	Het	gctactgaagtggCtcGG	gctactgaagtggCtcGA	cttcacatccctggggTtC
IWGSC_CSS_3B_scaff_10775915	Kronos4485	4701	C	T	Het	Het	ccaaggcgtcagagagG	ccaaggcgtcagagagA	agacctcacatGtccC
IWGSC_CSS_5AL_scaff_2754304	Kronos4485	2301	G	A	Het	Het	taaccTGccatcgccccG	taaccTGccatcgccccA	cattGccaggcaTgacT
IWGSC_CSS_5BL_scaff_10919959	Kronos4485	1867	C	T	Hom	Hom	gatcccttggagaagG	gatcccttggagaagA	tcttgtccgaaacatgtcA
IWGSC_CSS_7AS_scaff_4245431	Kronos4485	3402	G	A	Hom	Hom	aaggcgctgtgttc	aaggcgctgtgttc	agtaagtggAAcgtcaagatcaT
IWGSC_CSS_7BL_scaff_6667357	Kronos4485	641	C	T	Het	Het	gatcAgctgctcattcgagG	gatcAgctgctcattcgagA	ttccctgtcaattgtgccC

Appendix B

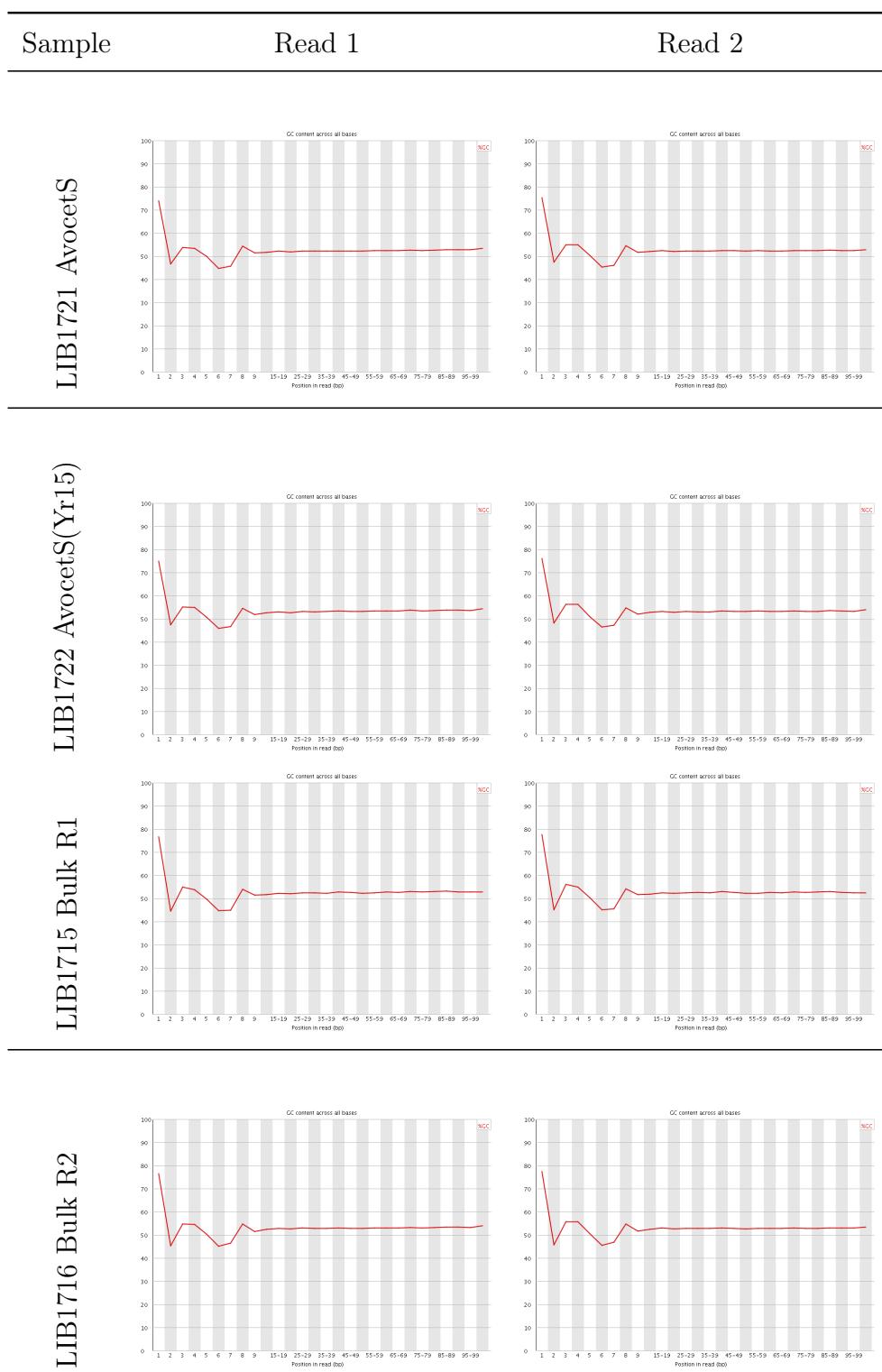
Quality Control

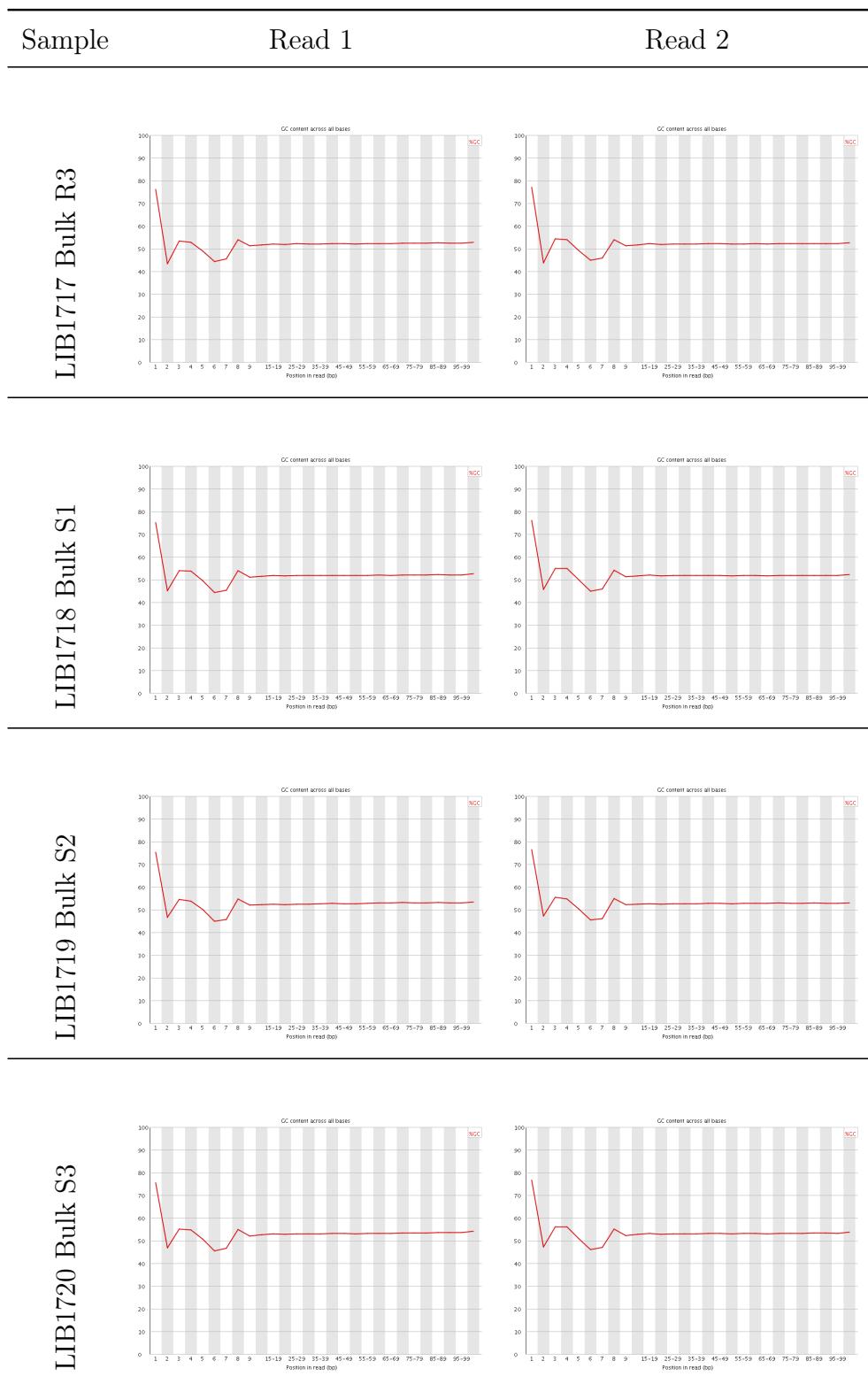
B.1 Sequence read quality





B.2 Sequence GC content





Appendix C

expVIP tutorial

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homonecloco / expvip-web

Code Issues 5 Pull requests 0 Projects 0 Wiki Pulse Graphs Settings

AllConcat

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](#).

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LoadingVM
RunKallisto
RunKallistoBatch
StartWebServer
Tutorial expVIP Graphical Interface (Wheat Expression Browser example)

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.

+ Add a custom sidebar

Clone this wiki locally

<https://github.com/homonecloco/expvip-web>

[Clone in Desktop](#)

Default data

The default values loaded in the virtual machine are available in this [link](#). These correspond to the wheat data from Borrill, Ramírez-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

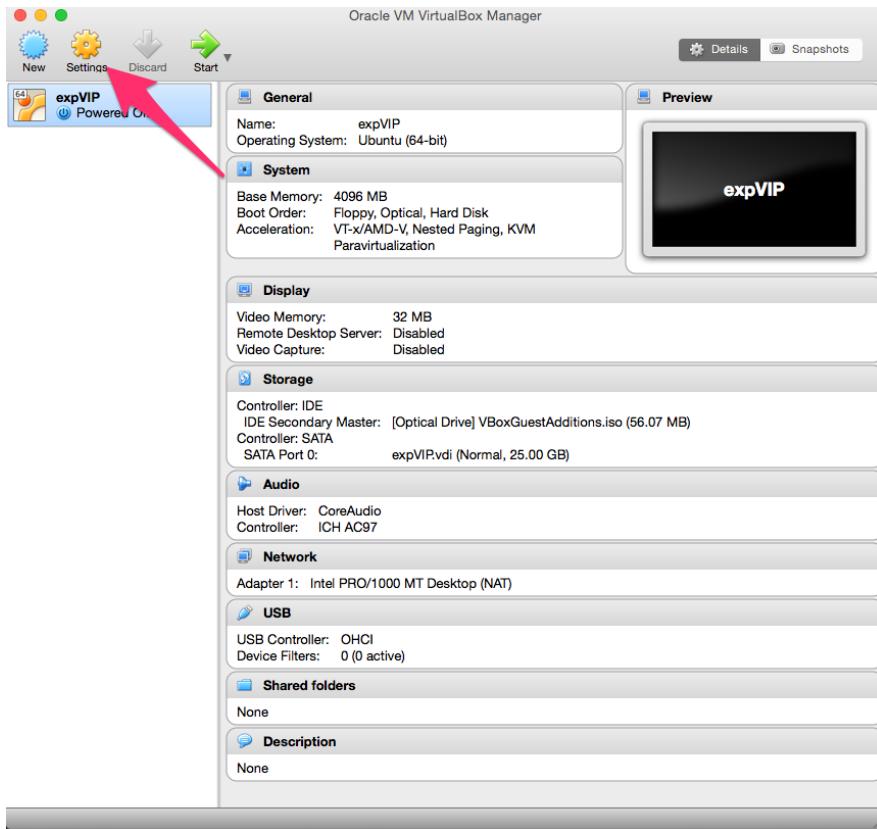
Download the `.ova` virtual machine and double click it. Virtual Box will open it. Accept the default options.

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

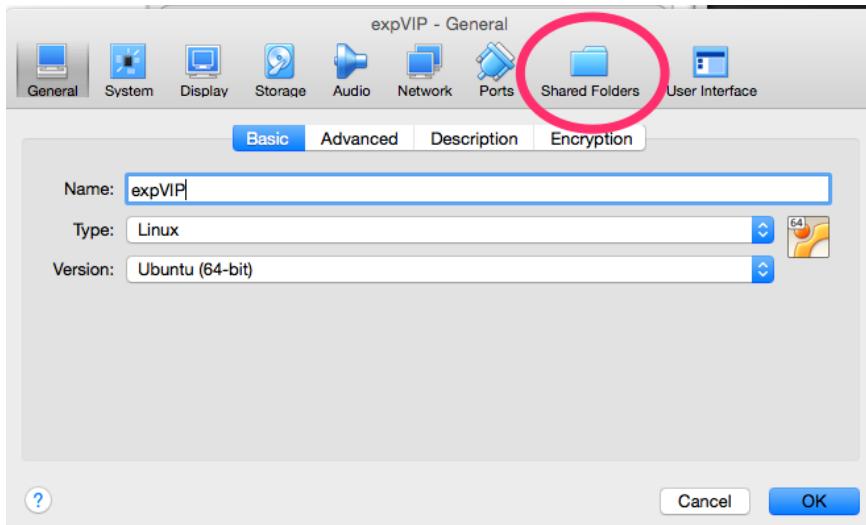
Available VMs:

- `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.

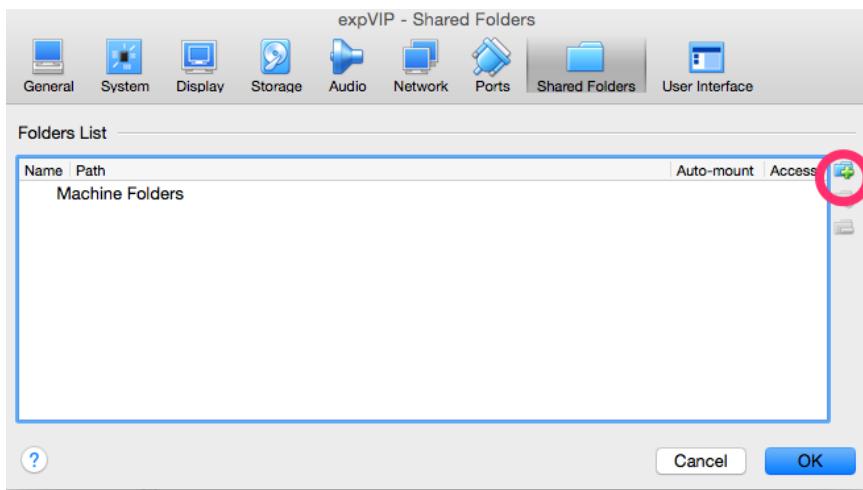
1. On the Oracle VM VirtualBox Manager select expVIP and click on the settings button



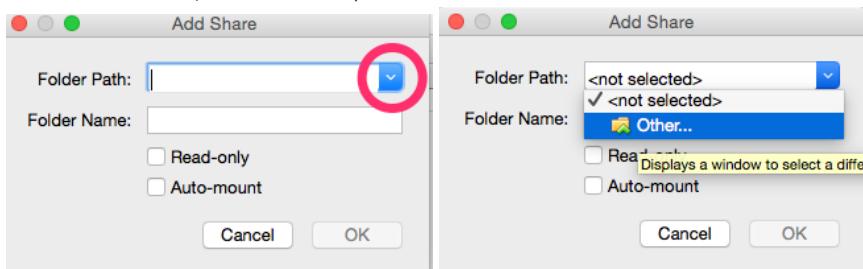
2. Click in Shared folders



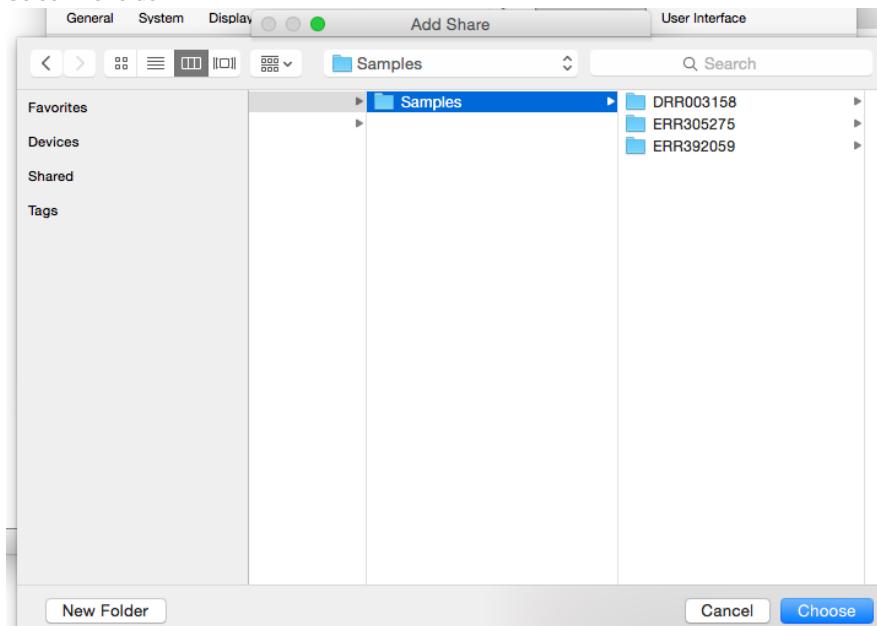
3. Add a new folder



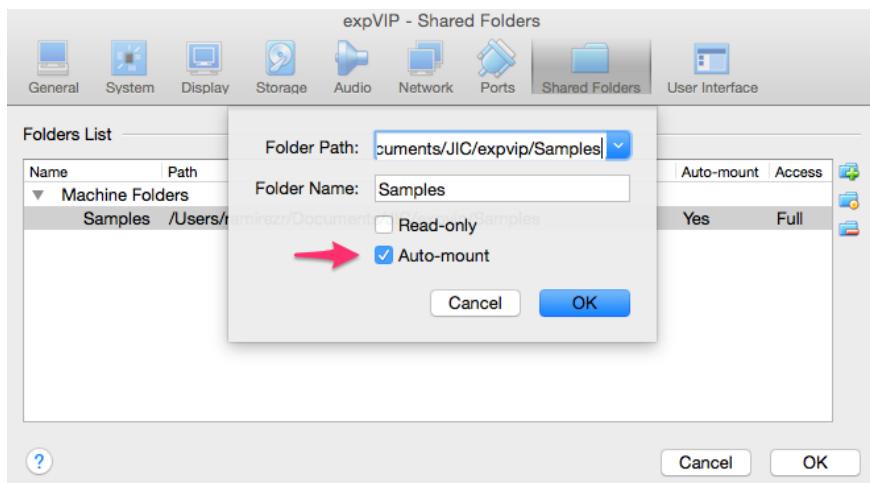
4. Search the Folder path with the experiments and the files with the metadata



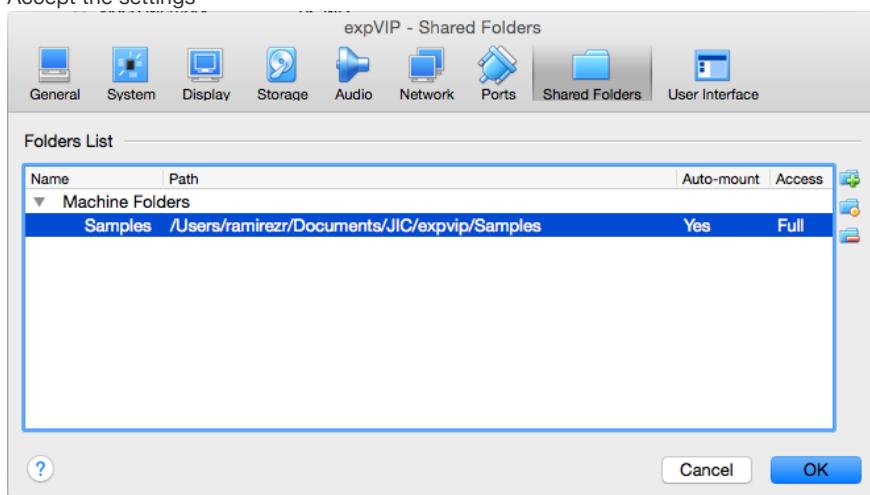
5. Select the folder



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

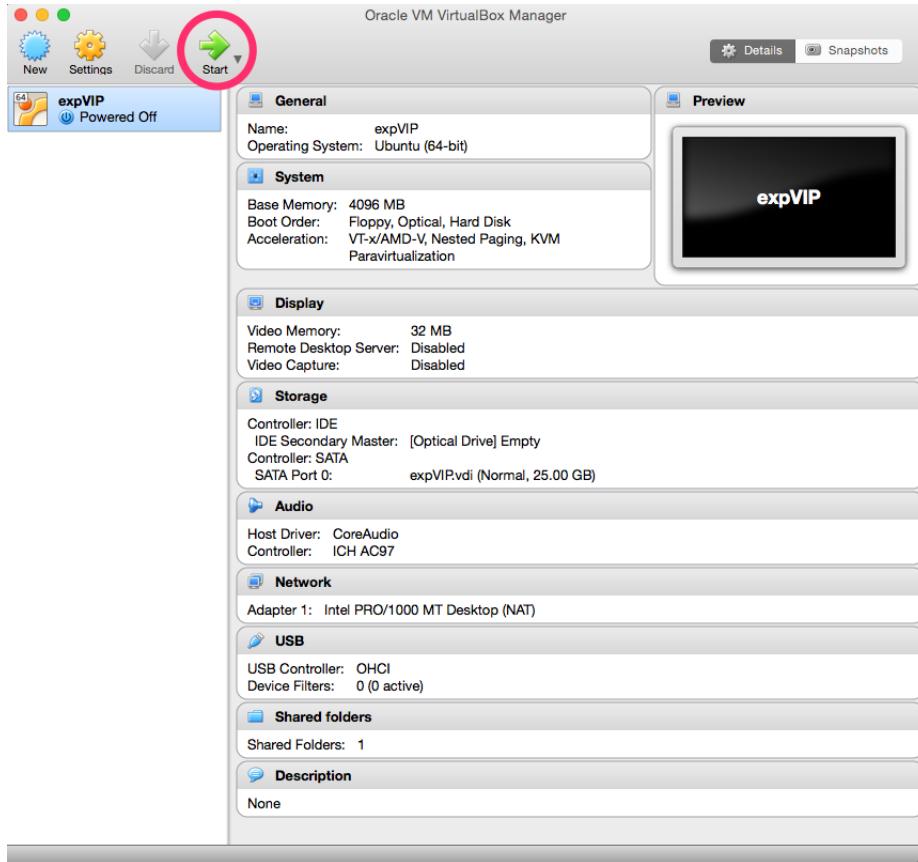


7. Accept the settings



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 necessary 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:

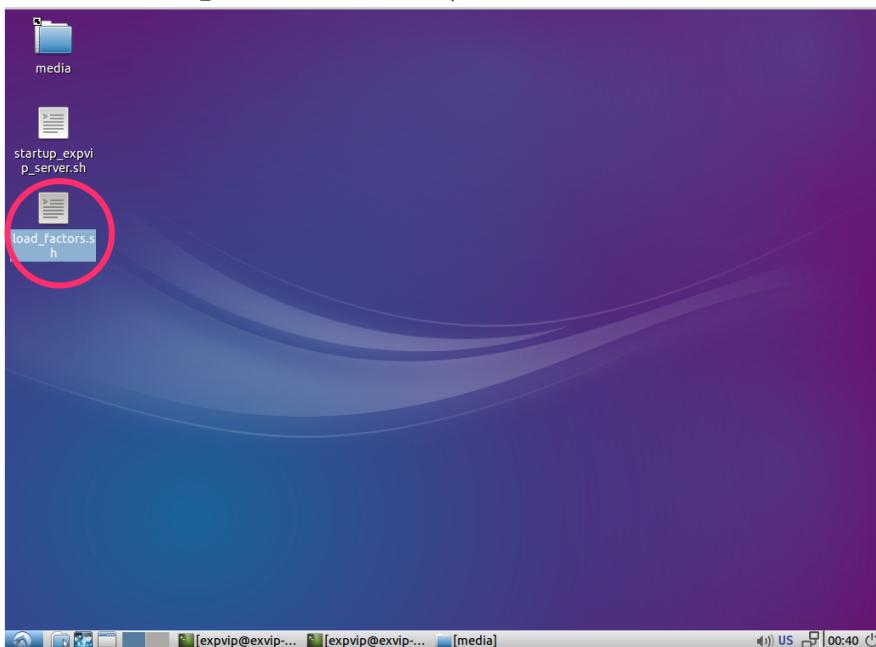
```

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
...

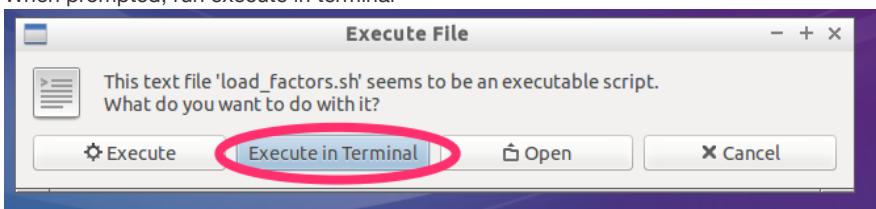
```

Wizard to load factors

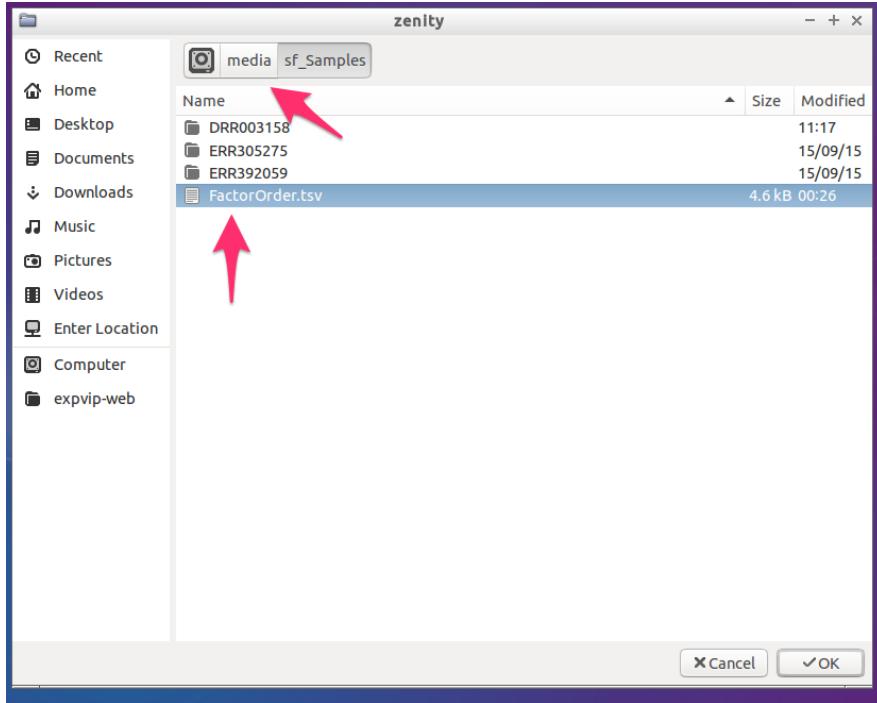
1. Double click in `load_factors.sh` in the desktop



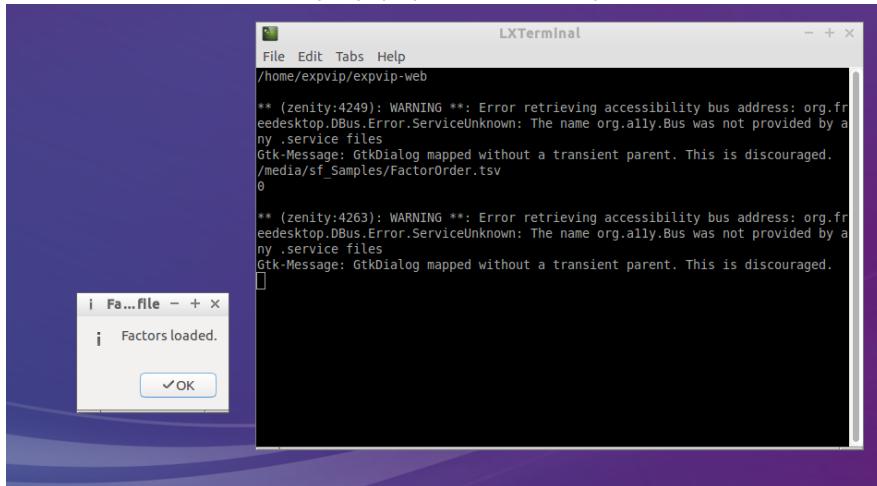
2. When prompted, run execute in terminal



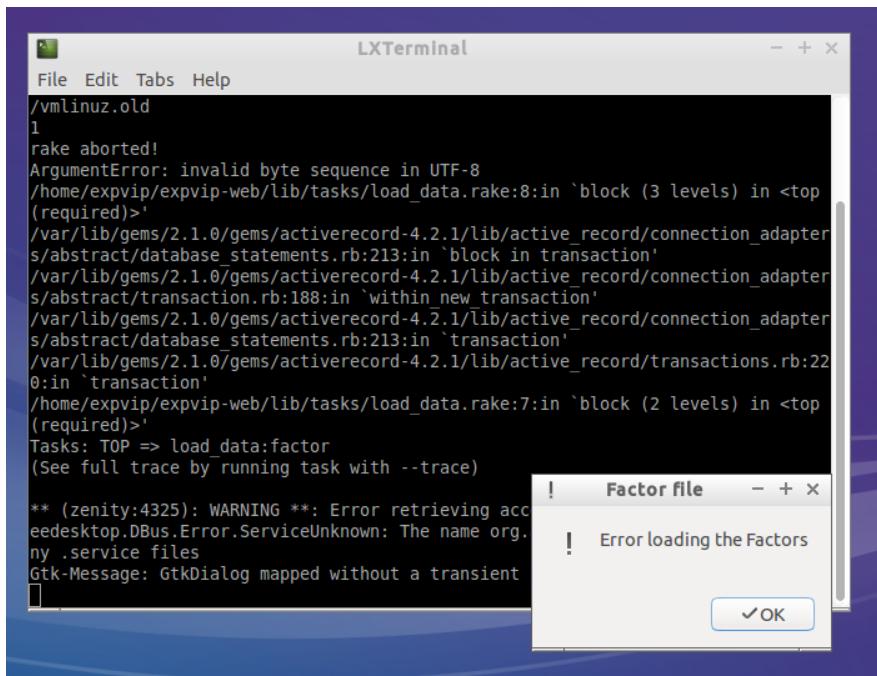
3. By default, the script goes to `/media`, which is the folder containing the shared folders that we have setup in the **LoadingVM** step.



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 `~/exvpip-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

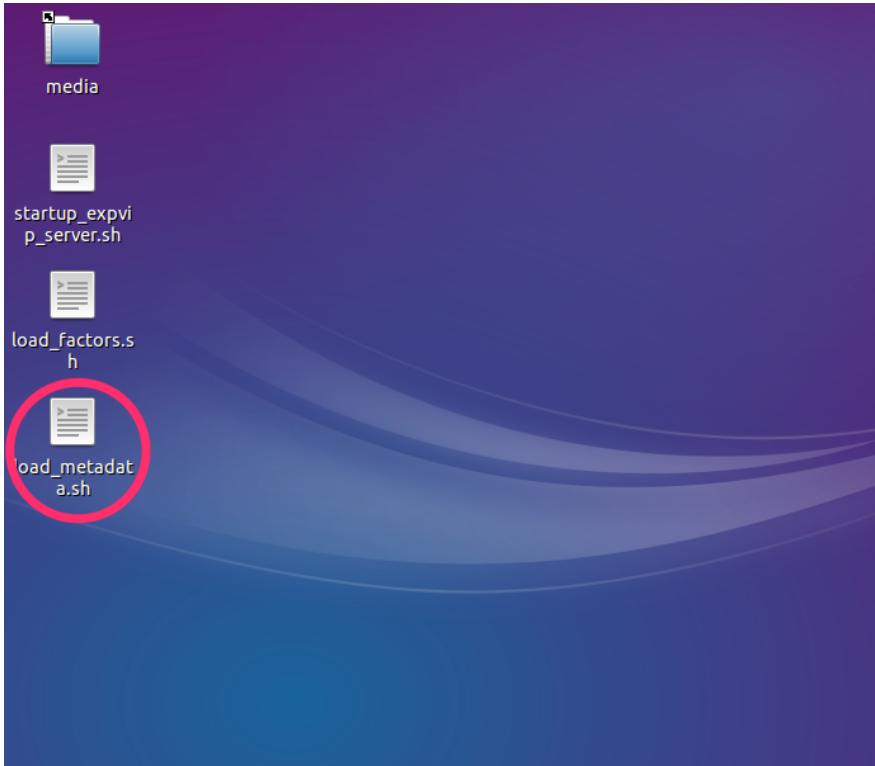
Important points

- 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.

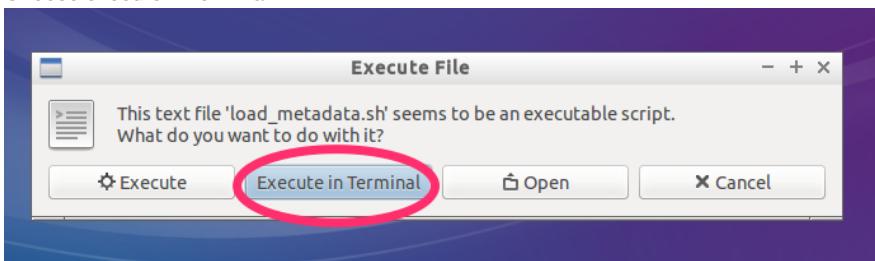
Using the graphical interface

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

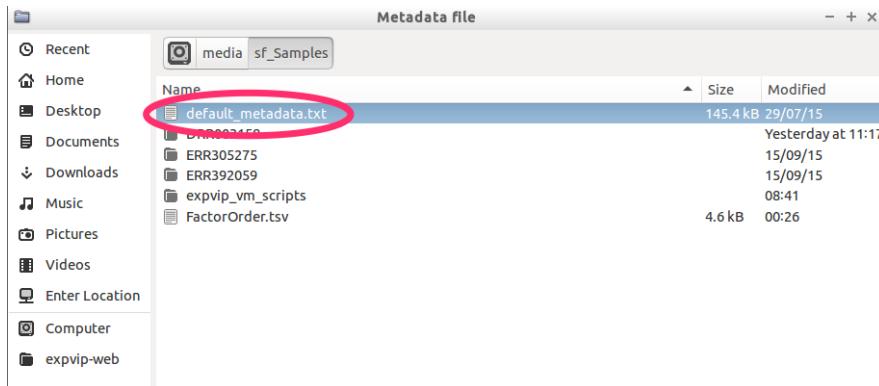
1. Double click on the `load_metadata.sh` icon in the desktop



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.

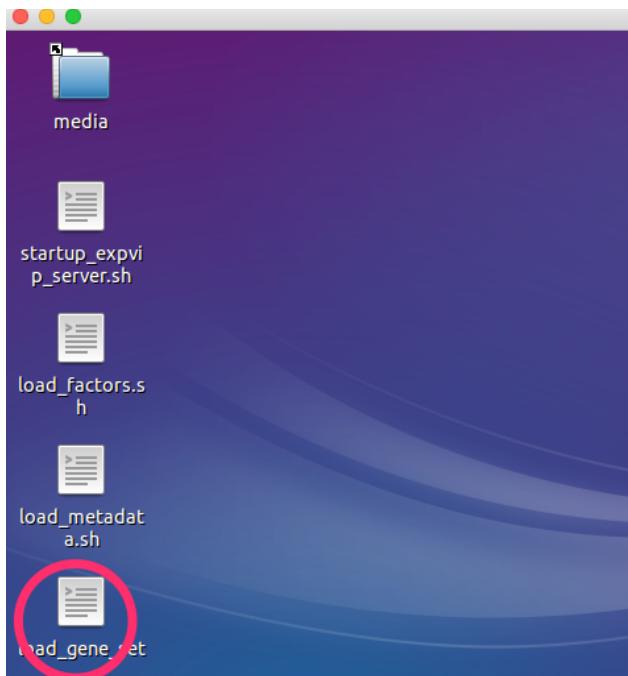
Besides the fasta file, it is necessary to give a name to the gene set. For this tutorial, the gene_set will be IWGSC2.26

Example fasta file

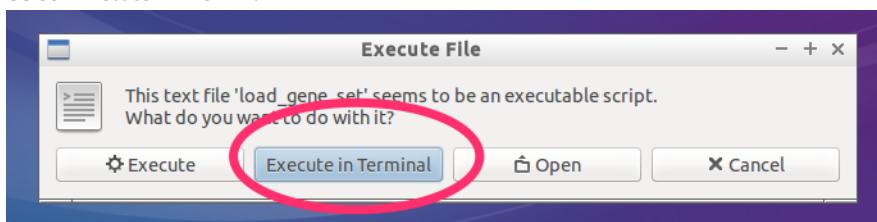
```
>Traes_5BL_3FC5BA305.1 cdna:novel scaffold:IWGSC2:IWGSC_CSS_5BL_scaff_1082268:5:199:-1 ge
TGCTGCTGCTAGGCTTGAAGAGGTTGCTGGCAAGCCTCCAGTCAGTCTGCTCGGCAGCTCATTC
GAGGGGCTGTGAGGAGTGCCCCAAGAACGAGGATGTTGGTTGAGGCATGCCGGTTGG
TAGCCAGATGAGTCAGTAATTGCCAGGGGTGTGAAGGCAATTCCAACCTCTGT
GAAGCTGTGGCTGCA
>Traes_6BL_9BB648D51.1 cdna:novel scaffold:IWGSC2:IWGSC_CSS_6BL_scaff_430516:302:1741:-1
TCCCTATCTGTTCTTGCGACTCCCTGATCCAATCGATCCATCAGGGCTCGACTAACT
TCTTCAGCGCTCTTCAAGCGGGAGATCTACCAGCTGGCGGAGGGGGCTAGGTGCA
GGCGTGCAGCCAAAGTCCGCACCCGGCTCTAGGTTCTGCTAATCTTCTCCACCTGTGA
TACGCGCTCCGGGCTAGGAGCACTCGTTGCCGGCTGCCCTCGTCGGAATGGCGGATG
```

Graphical interface

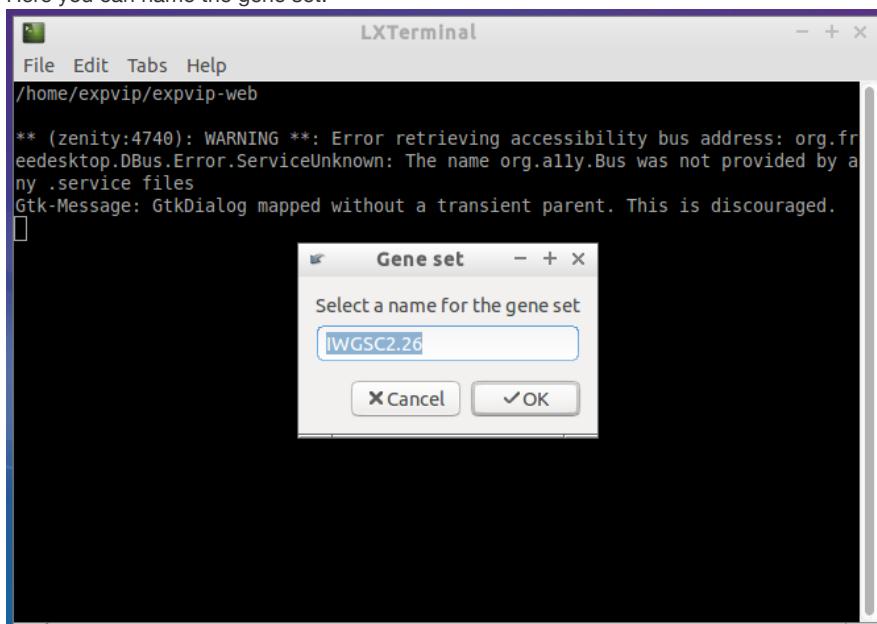
1. Double click on the `load_gene_set` script



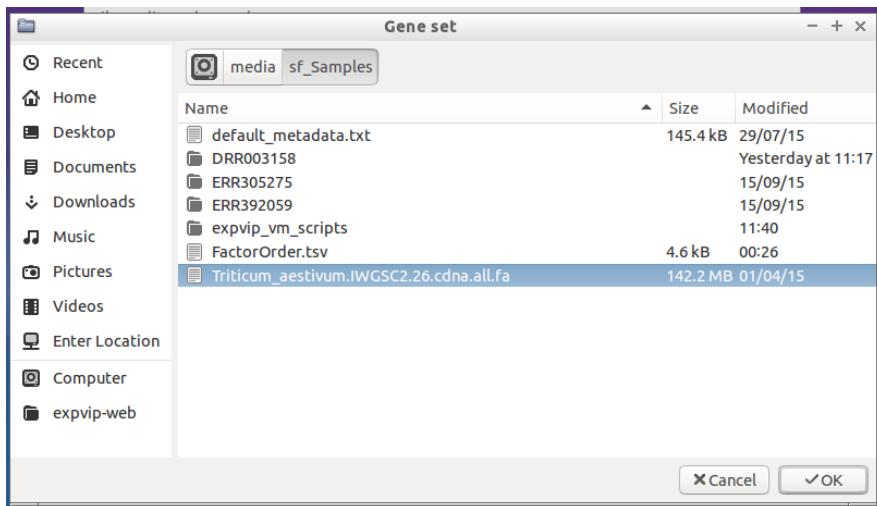
2. Select Execute in Terminal



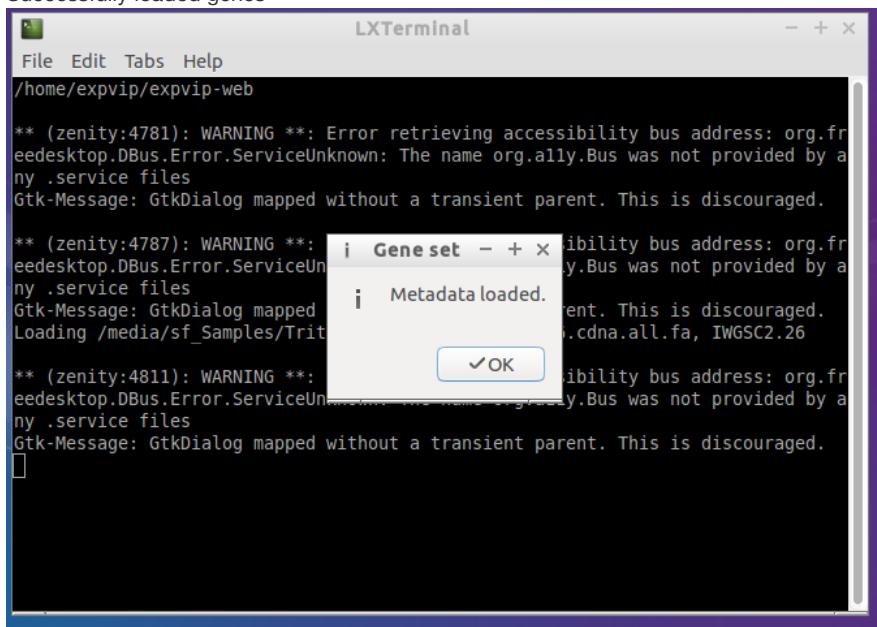
3. Here you can name the gene set.



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_2AL_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 generated 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

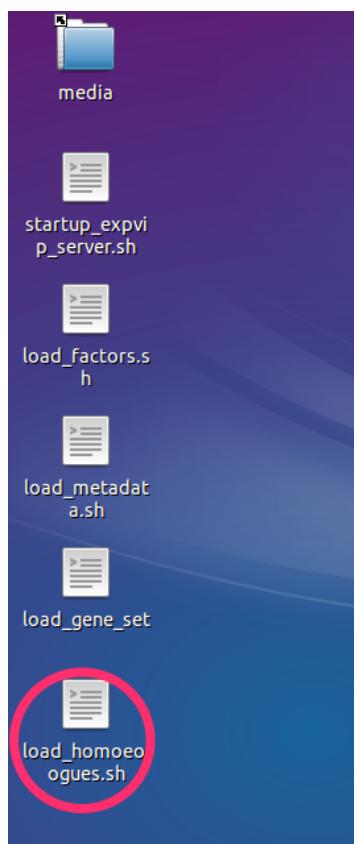
FROM
    homology_member
INNER JOIN homology USING (homology_id)
INNER JOIN method_link_species_set USING (method_link_species_set_id)
INNER JOIN gene_member USING (gene_member_id)
WHERE method_link_species_set.name="T.aes homoeologues";
```

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

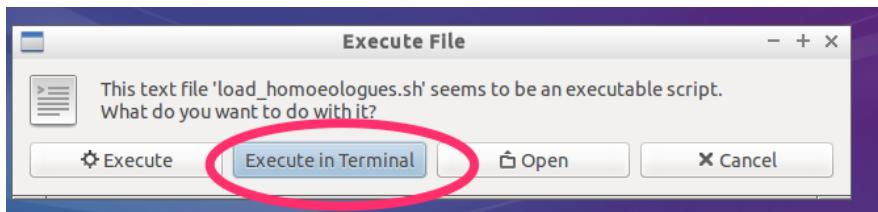
```
ruby bin/homologyTable.rb compara_homology.txt homology.txt homology_counts.txt
```

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

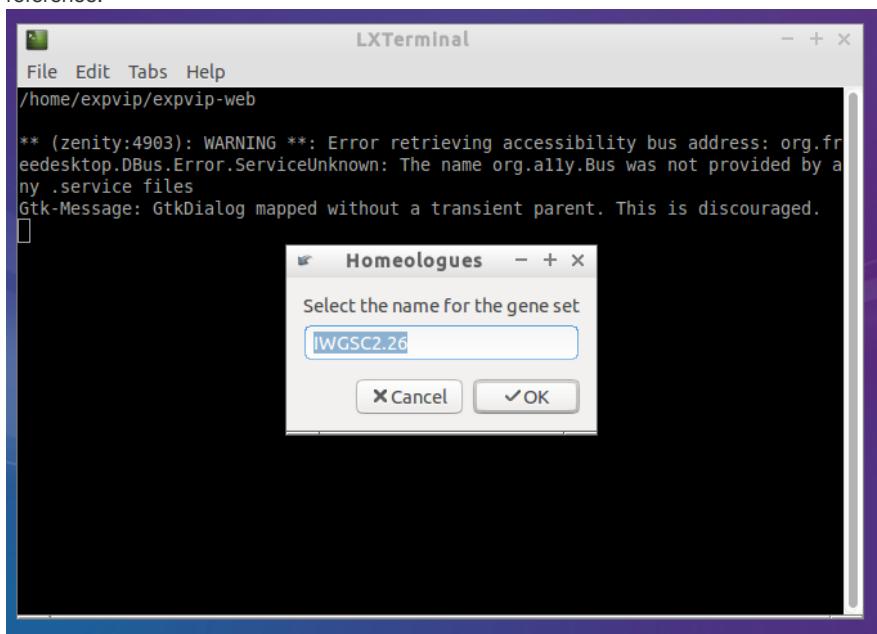
At this point, the homoloegues are called A,B and D. This is going to change on a future release to allow any chromosome group naming.



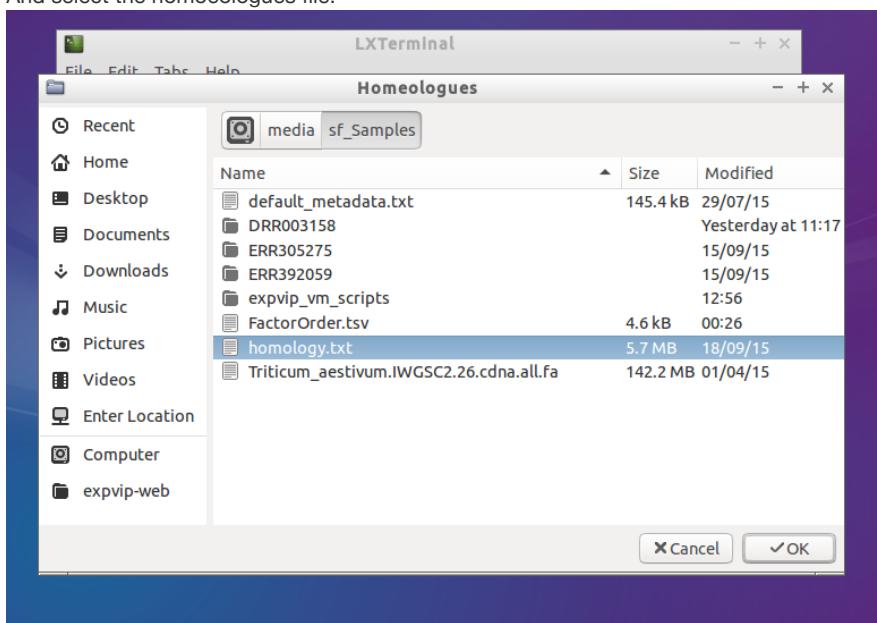
1. Double click on the `load_homoeologues.sh` script
2. Select Execute in Terminal



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

```

File Edit Tabs Help
ny .service files
Gtk-Message: GtkDialog mapped without a transient parent. This is discouraged.

** (zenity:5266): WARNING **: Error retrieving accessibility bus address: org.freedesktop.DBus.Error.ServiceUnknown: The name org.ally.Bus was not provided by any .service files
Gtk-Message: GtkDialog mapped without a transient parent. This is discouraged.
Loading /media/sf_Samples/home/allybus/IWGSC2.26/.service
{:gene_set=>"IWGSC2.26", :file=>"/home/allybus/IWGSC2.26/homology.txt"}
Loaded 103274 genes in memory
Loaded 10000 Homologies (Trae...
Loaded 20000 Homologies (Trae...
Loaded 30000 Homologies (Trae...
Loaded 40000 Homologies (Trae...
Loaded 50000 Homologies (Trae...
Loaded 60000 Homologies (Traes_5DL_7B922D9F7)
Loaded 70000 Homologies (Traes_2BS_7A9FD08C2)
Loaded 79349 Homologies

** (zenity:5279): WARNING **: Error retrieving accessibility bus address: org.freedesktop.DBus.Error.ServiceUnknown: The name org.ally.Bus was not provided by any .service files
Gtk-Message: GtkDialog mapped without a transient parent. This is discouraged.

```

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:

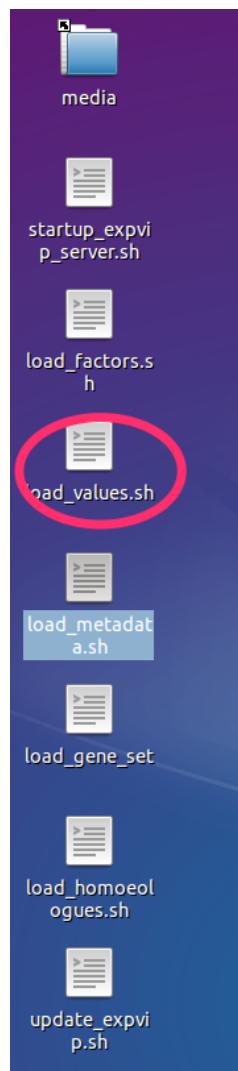
1. Load the precomputed expression values into the database, or
2. Run kallisto to generate the expression data. These are then loaded directly into the database.

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 must 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.

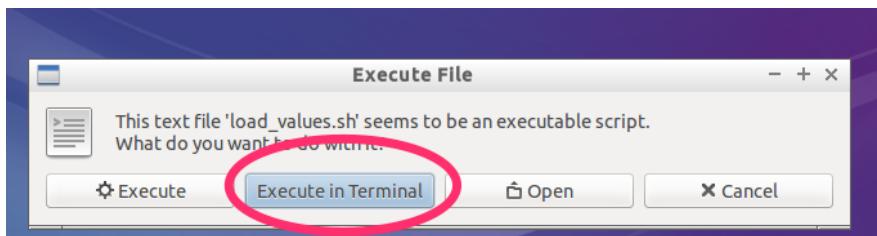
For the case of wheat in which we have already generated the kallisto mapping of 418 RNA-seq studies, the expression table can be downloaded directly from [here](#). The `.txt` files are called `final_output_counts.txt` and `final_output_tpm.txt` for the corresponding expression unit.

Loading from the script

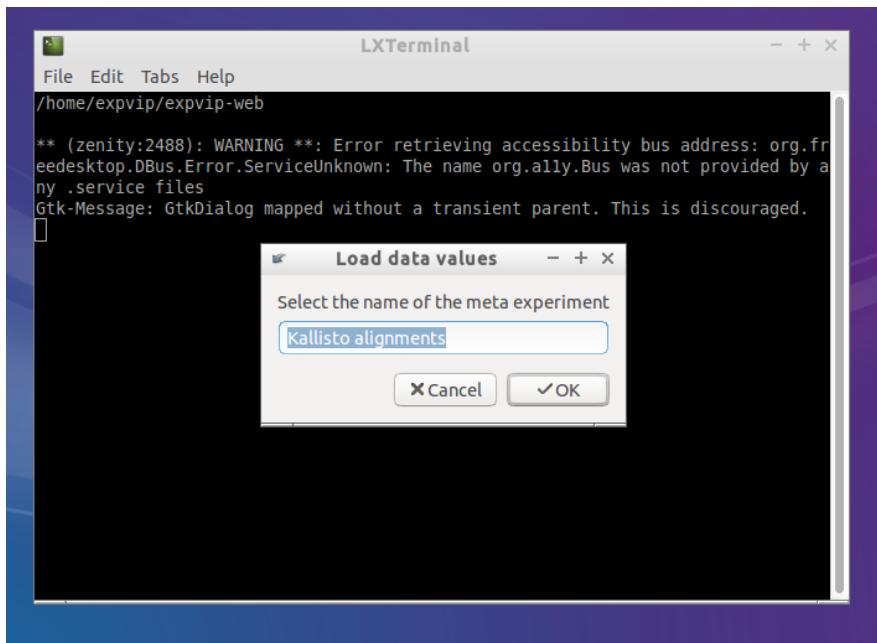


1. Double click on the `load_values.sh`

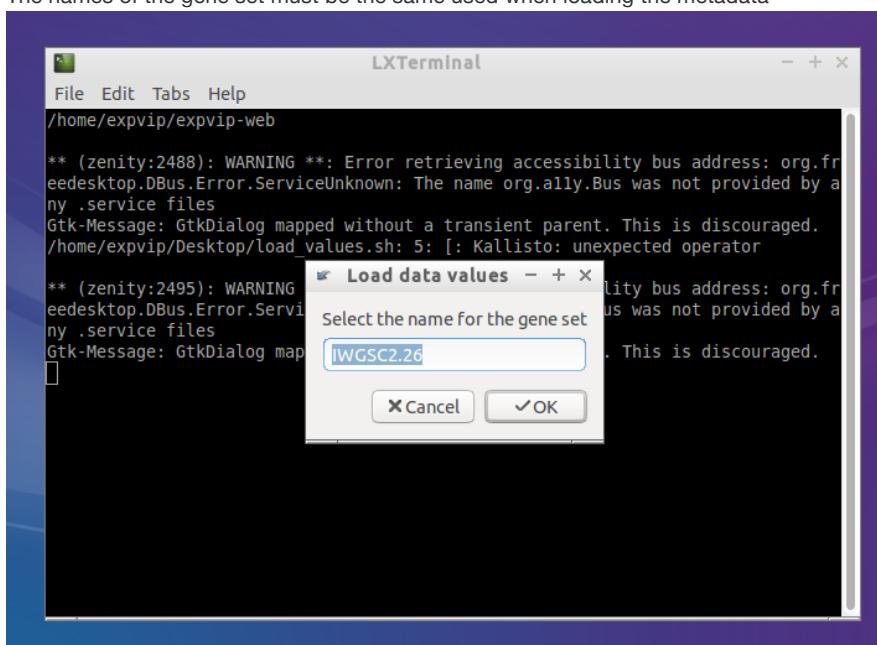
2. Click one Execute in Terminal



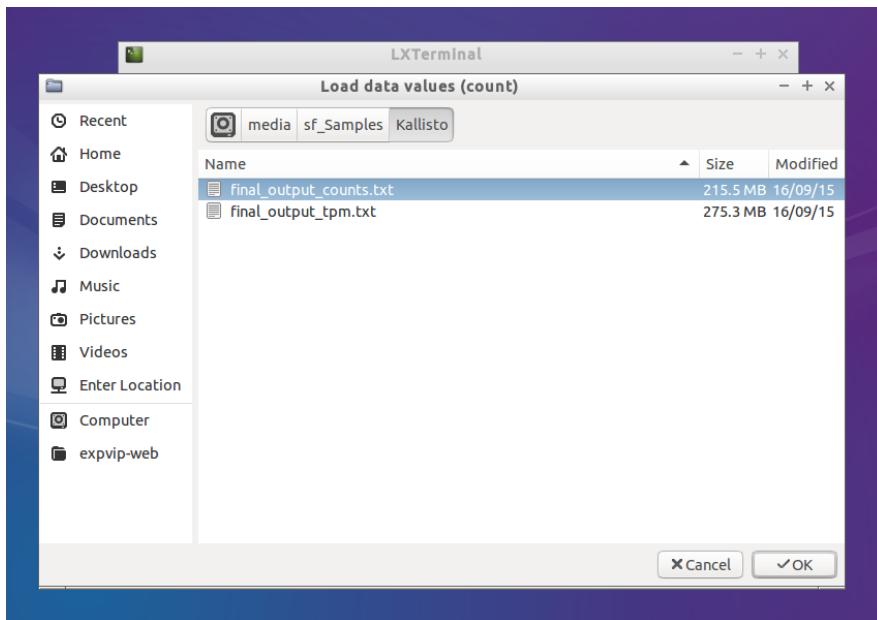
3. Select a name for the set of alignments. expVIP can keep several runs of alignments in the database. The ability to select between them will be added in a future release.



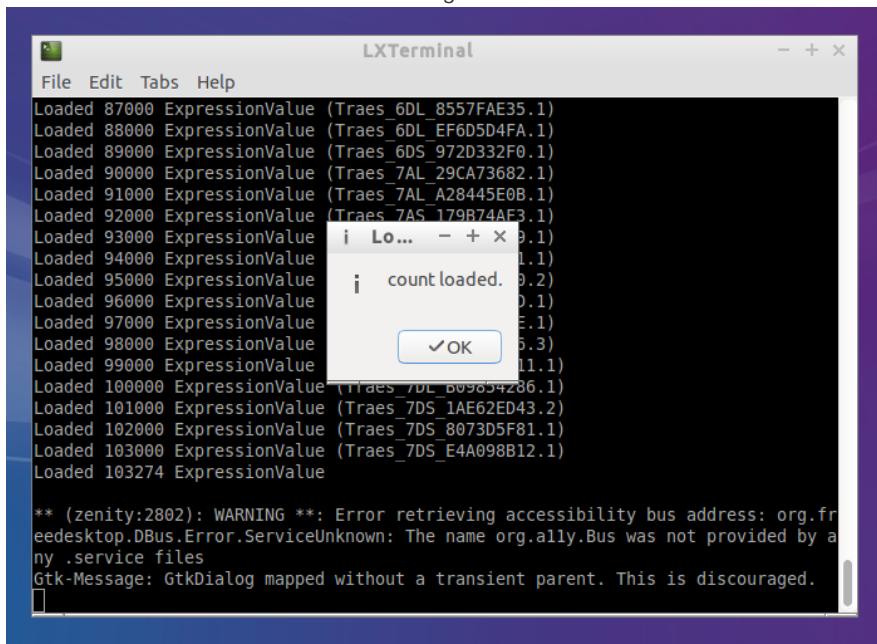
4. The names of the gene set must be the same used when loading the metadata



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:

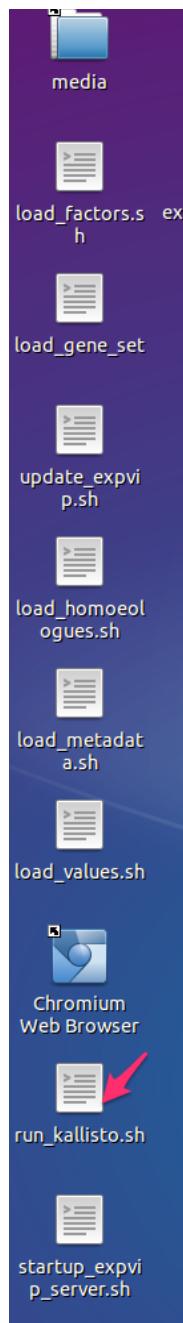
```
rake kallisto:runAndStorePaired[Index, folder/with/samples/ACCESSION, experiment_title, IWGS
```

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

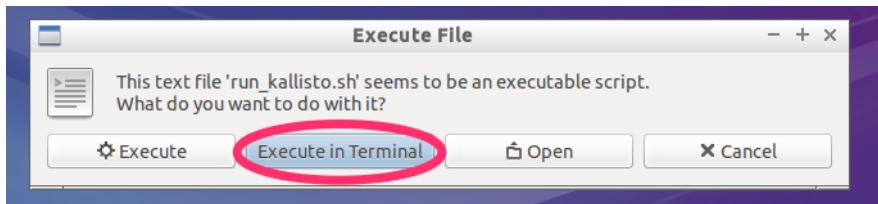
expVIP can run `Kallisto` and load the `tpm` and `counts` to the database. The only requirement is to run `kallisto index` on the transcriptome reference.

Graphical interface

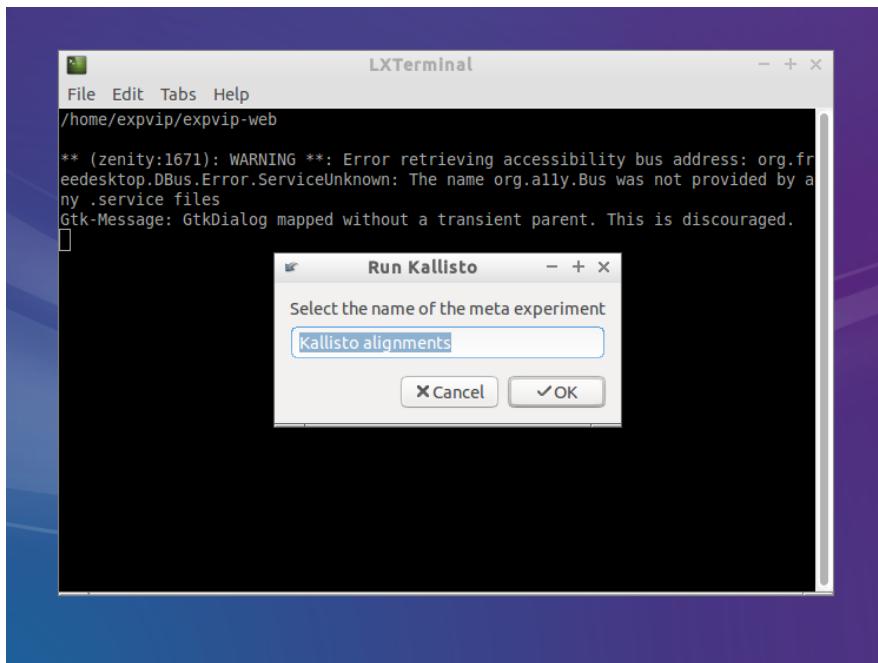


1. Double click on run_kallisto.sh

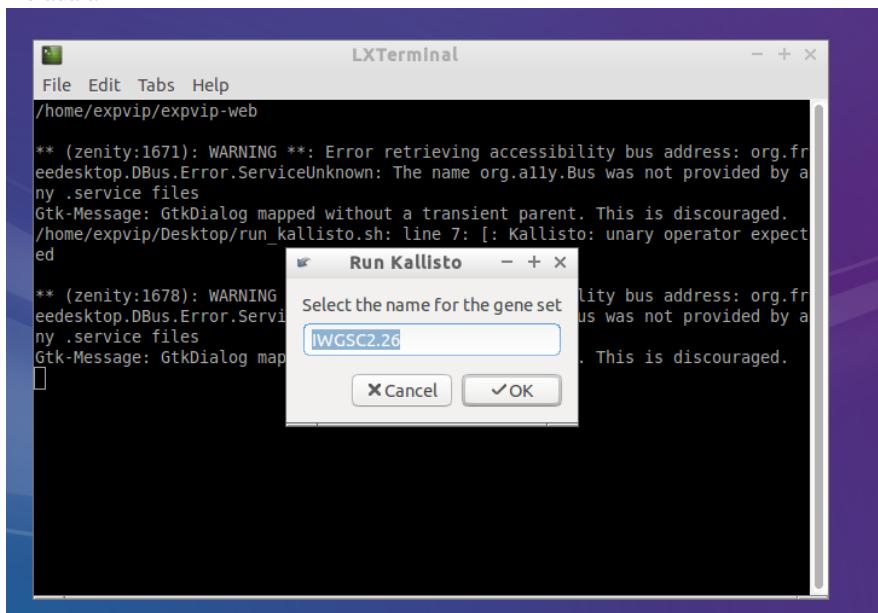
2. Click on Execute on terminal



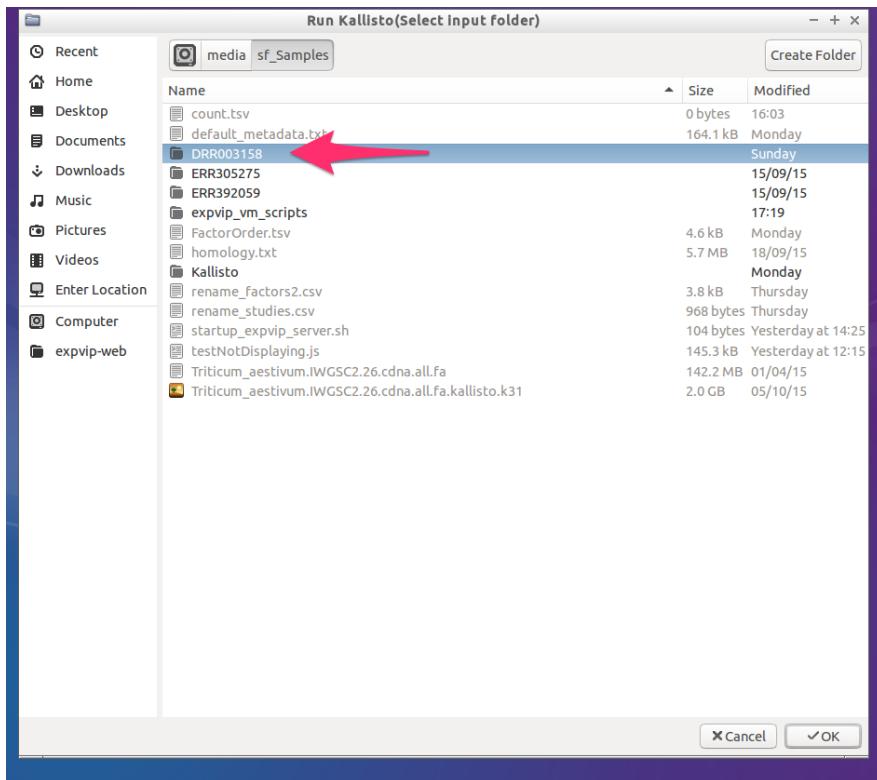
3. Give a name to the set of mappings to be grouped. All mappings done with the same reference and preference should have the same name.



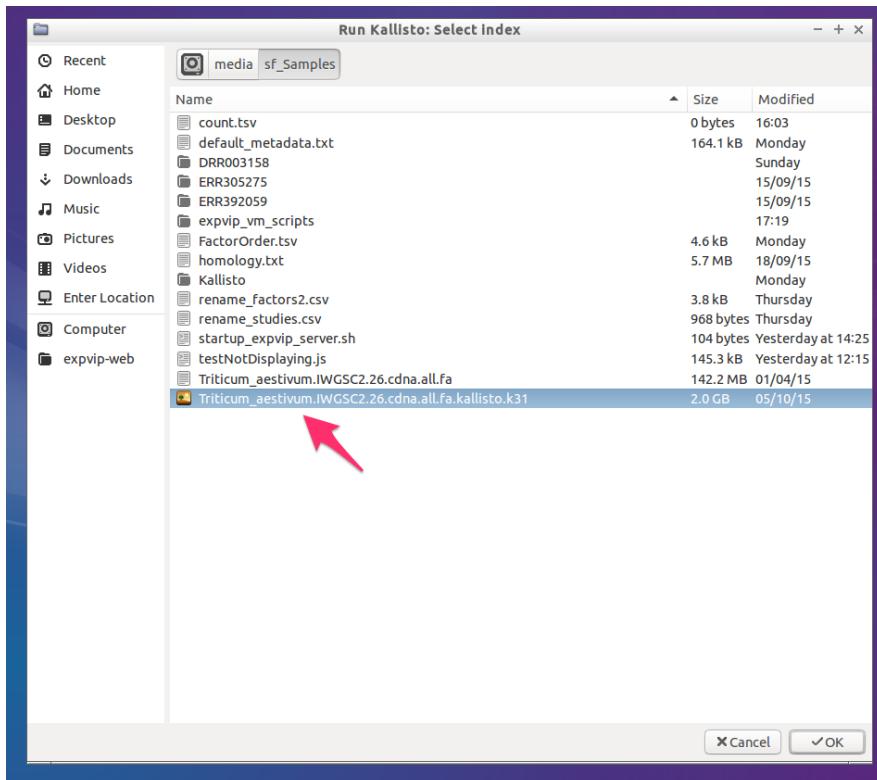
4. Get the name of the reference. This name must be the same used when loading the metadata



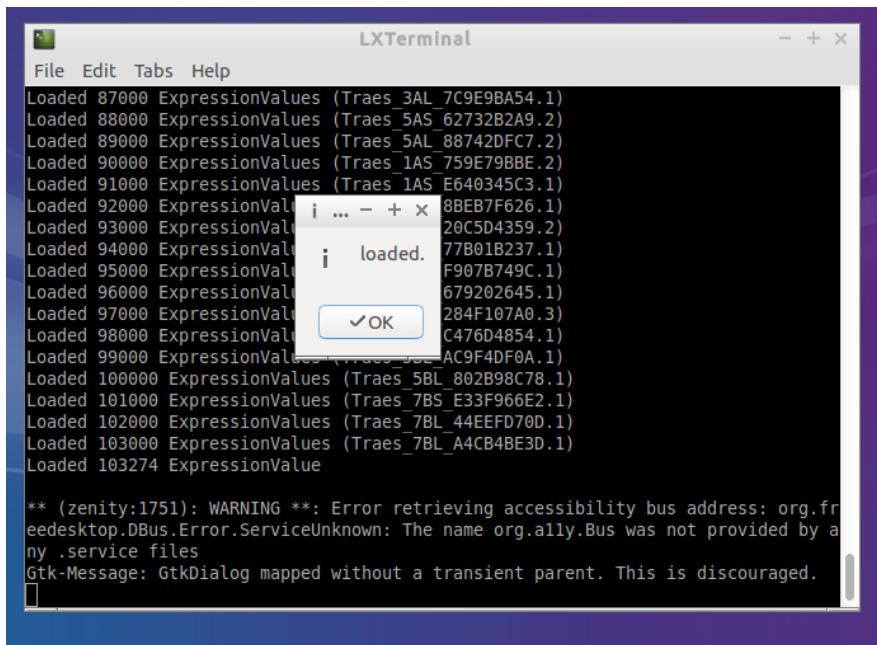
5. Select a folder with the reads. The reads must be paired reads. The folder name must be the same as the accession used on the metadata.



6. Select the kallisto index



7. Wait for Kallisto to run and load the data



You can repeat this with all the samples or you can use the [batch load](#).

Rake task

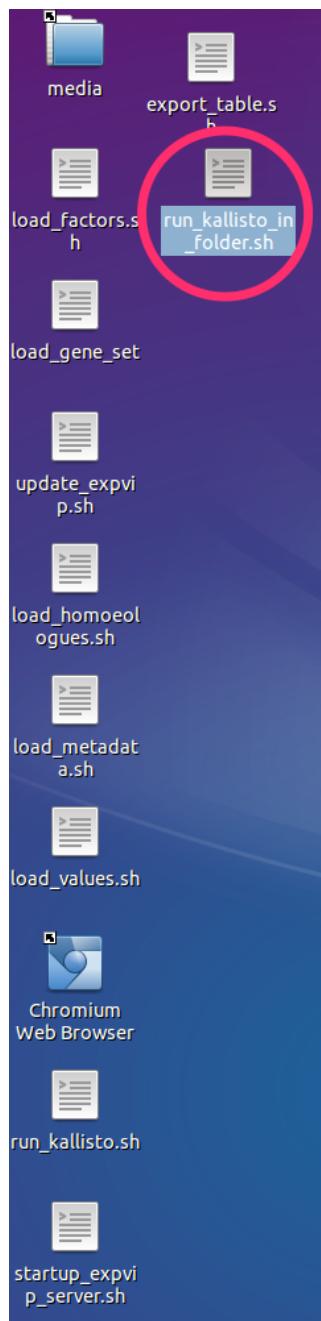
```
kallisto:runAndStorePaired[kallistoIndex,input_folder,metaExperimentName,geneSetName]
```

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

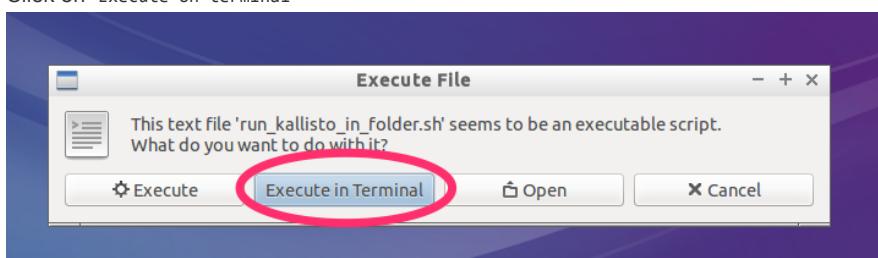
expVIP can run `Kallisto` and load the `tpm` and `counts` to the database from multiple samples. The only requirement is to run `kallisto index` on the transcriptome reference.

Graphical interface

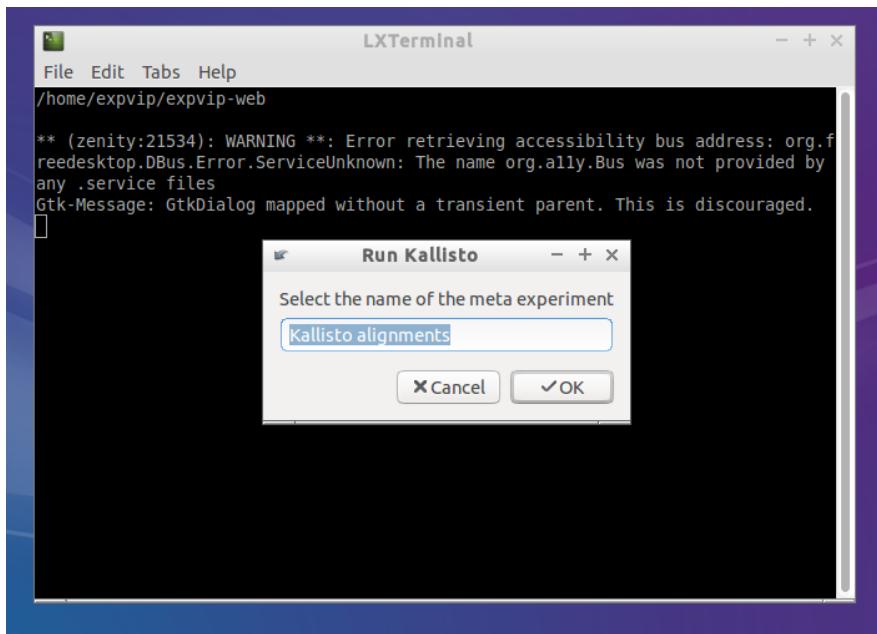


1. Double click on run_kallisto.sh

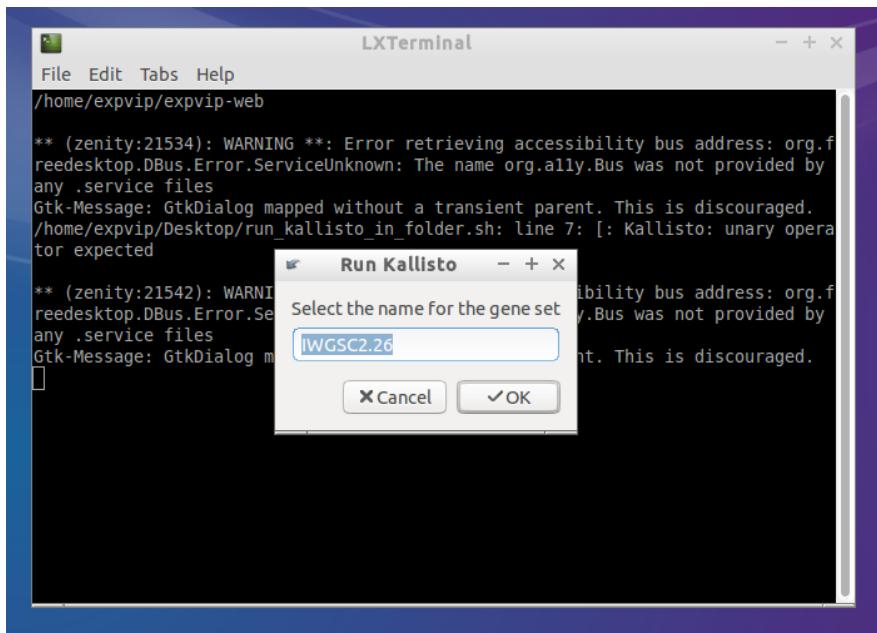
2. Click on Execute on terminal



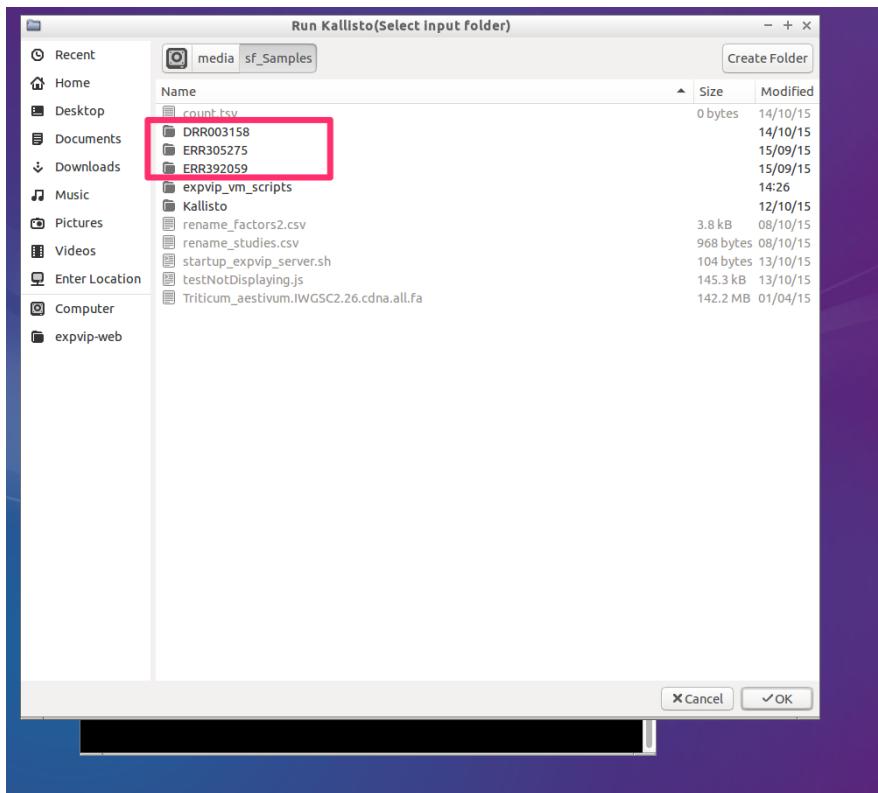
3. Give a name to the set of mappings to be grouped. All mappings done with the same reference and preference should have the same name.



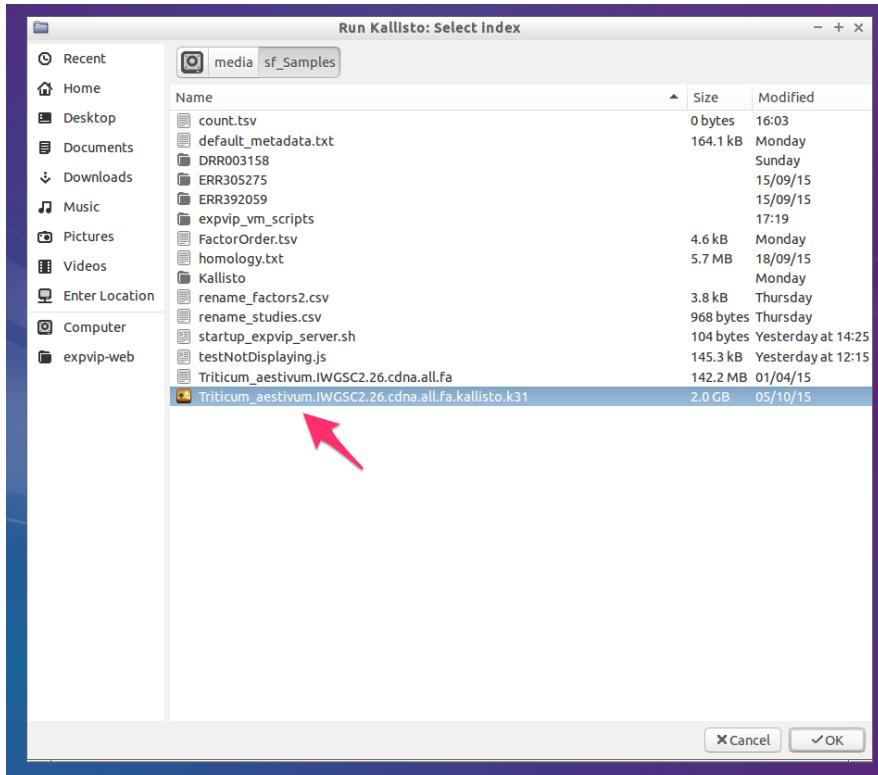
4. Get the name of the reference. This name must be the same used when loading the metadata



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

```

Gtk-Message: GtkDialog mapped without a transient parent. This is discouraged.
/home/expvip/Desktop/run_kallisto_in_folder.sh: line 7: [: Kallisto: unary operator expected

** (zenity:4398): WARNING **: Error retrieving accessibility bus address: org.freedesktop.DBus.Error.ServiceUnknown: The name org.ally.Bus was not provided by any .service files
Gtk-Message: GtkDialog mapped without a transient parent. This is discouraged.

** (zenity:4402): WARNING **: Error retrieving accessibility bus address: org.freedesktop.DBus.Error.ServiceUnknown: The name org.ally.Bus was not provided by any .service files
Gtk-Message: GtkDialog mapped without a transient parent. This is discouraged.

** (zenity:4411): WARNING **: Error retrieving accessibility bus address: org.freedesktop.DBus.Error.ServiceUnknown: The name org.ally.Bus was not provided by any .service files
Gtk-Message: GtkDialog mapped without a transient parent. This is discouraged.

** (zenity:4436): WARNING **: Error retrieving accessibility bus address: org.freedesktop.DBus.Error.ServiceUnknown: The name org.ally.Bus was not provided by any .service files
Gtk-Message: GtkDialog mapped without a transient parent. This is discouraged.

```

A small modal dialog box is overlaid on the terminal window, displaying the text "i ... - + x" and "loaded." with a blue "OK" button.

Repeat this with all the samples.

Rake task

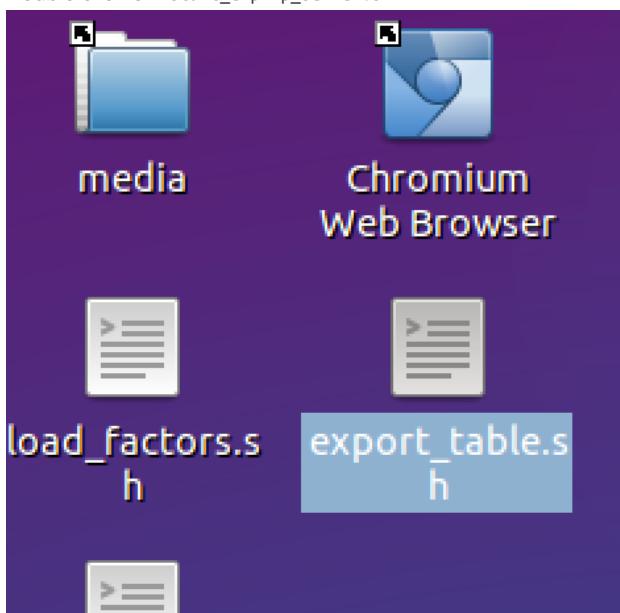
```
kallisto:runAndStorePairedFolder[kallistoIndex,input_folder,metaExperimentName,geneSetNameName]
```

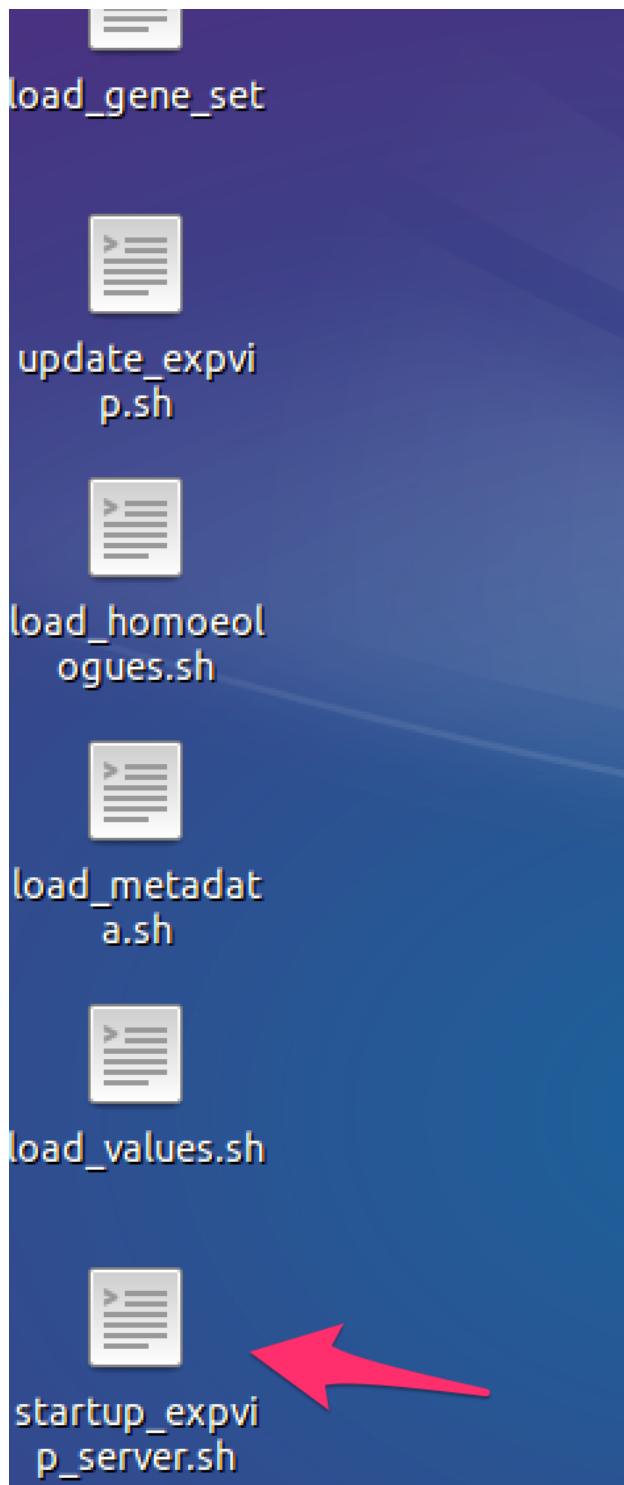
Where `metaExperimentName` is the name of the group of alignments under the same conditions and ``geneSetName`` is the name of the reference.

Starting expVIP web server

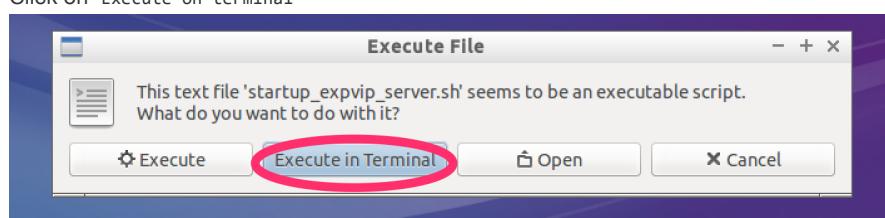
Once the data is loaded, you can visualize the the expression in the expVIP virtual machine.

1. Double click on `start_exvip_server.sh`



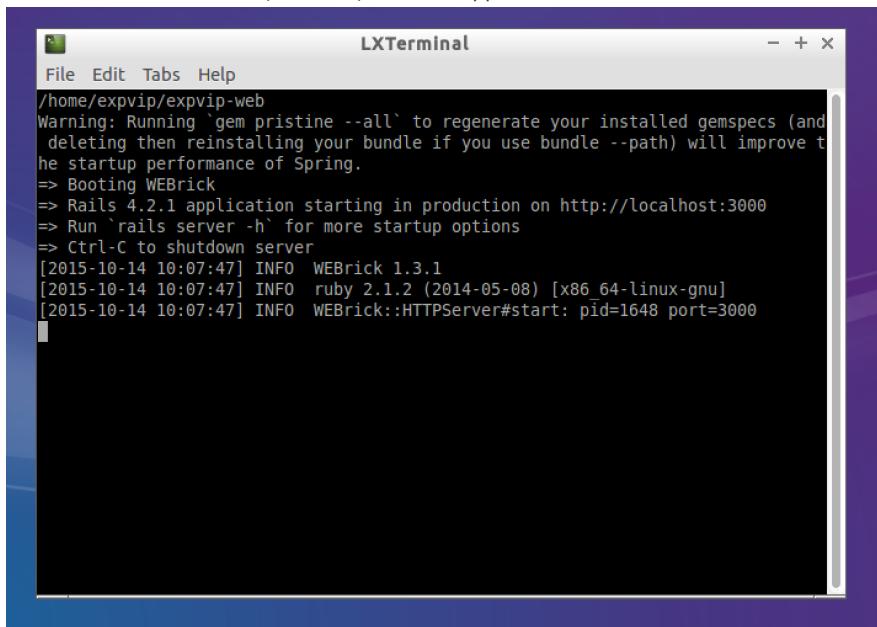


2. Click on Execute on terminal



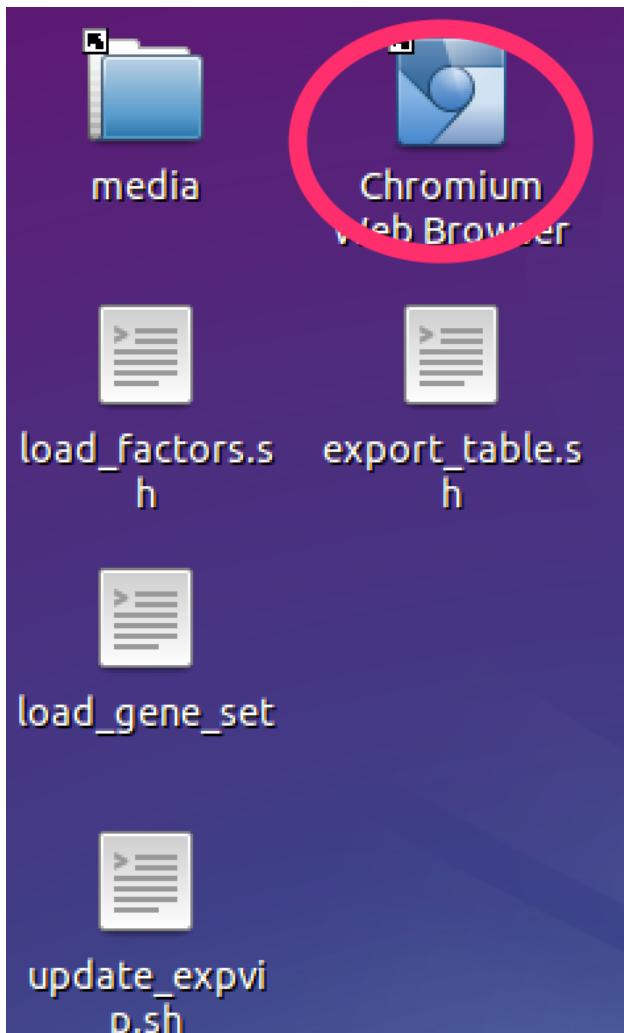
3. Wait for the webserver to start. You know it is ready when the line

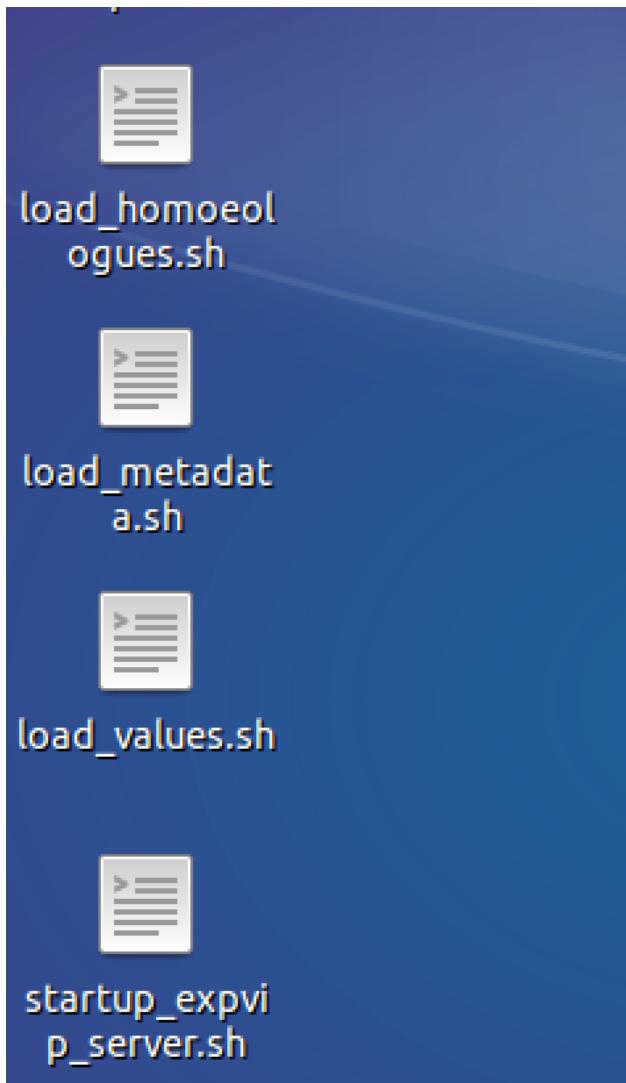
WEBrickHTTPServer#start: pid=xxxx port=3000 appears in the console



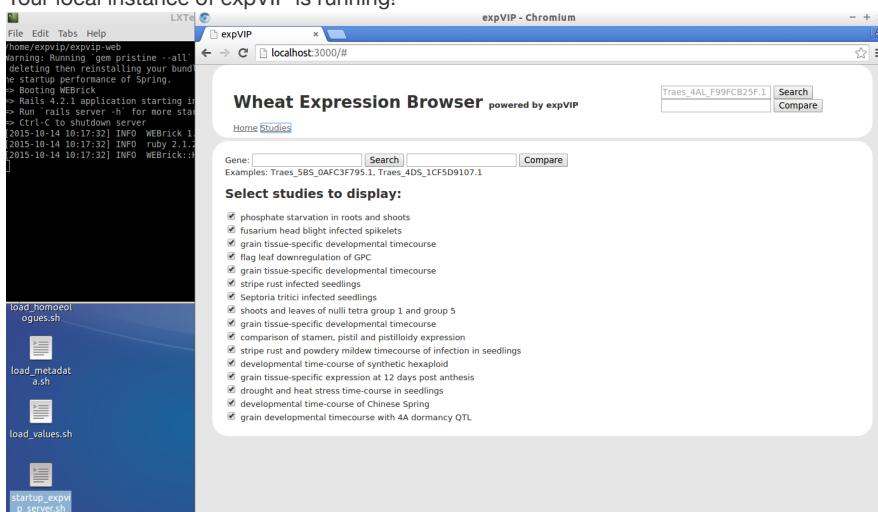
```
LXTerminal
File Edit Tabs Help
/home/expvip/expvip-web
Warning: Running `gem pristine --all` to regenerate your installed gemspecs (and
deleting them reinstalling your bundle if you use bundle --path) will improve t
he startup performance of Spring.
=> Booting WEBrick
=> Rails 4.2.1 application starting in production on http://localhost:3000
=> Run `rails server -h` for more startup options
=> Ctrl-C to shutdown server
[2015-10-14 10:07:47] INFO  WEBrick 1.3.1
[2015-10-14 10:07:47] INFO  ruby 2.1.2 (2014-05-08) [x86_64-linux-gnu]
[2015-10-14 10:07:47] INFO  WEBrick::HTTPServer#start: pid=1648 port=3000
```

4. Double click in Chromium Web Broser.





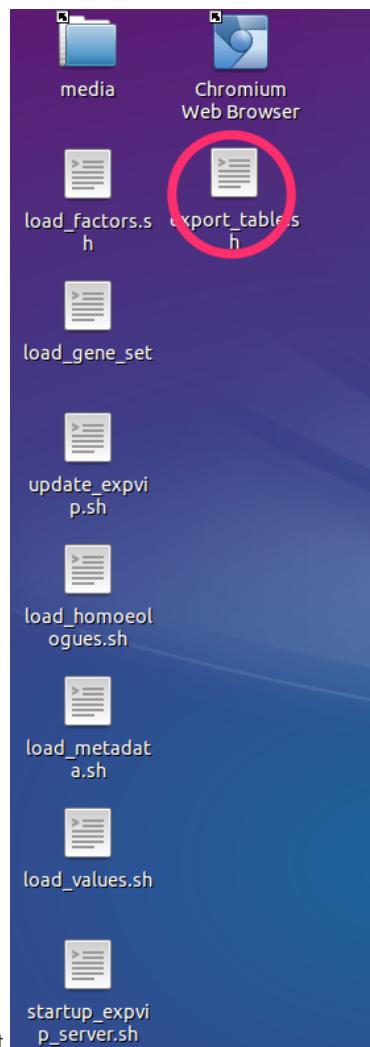
5. Your local instance of expVIP is running!



Export data

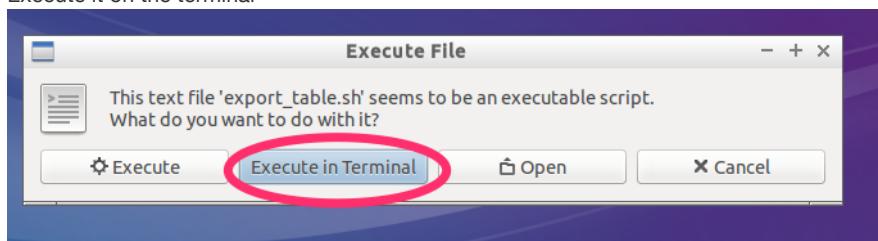
The data loaded in `expVIP` can be exported to be run in DESeq2, or any other software to do differential expression analysis.

Wizard to export data

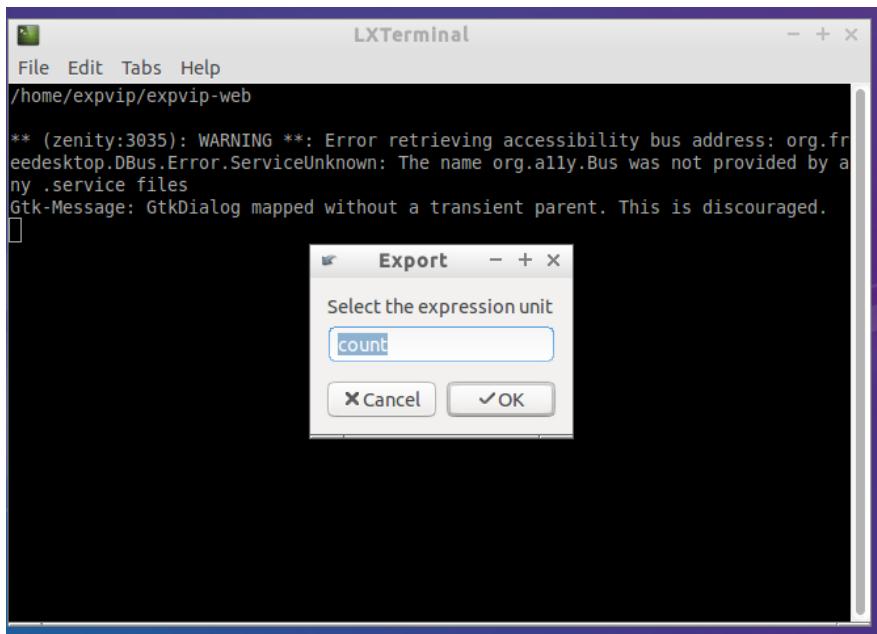


1. Double click on the the `export_tables.sh` script

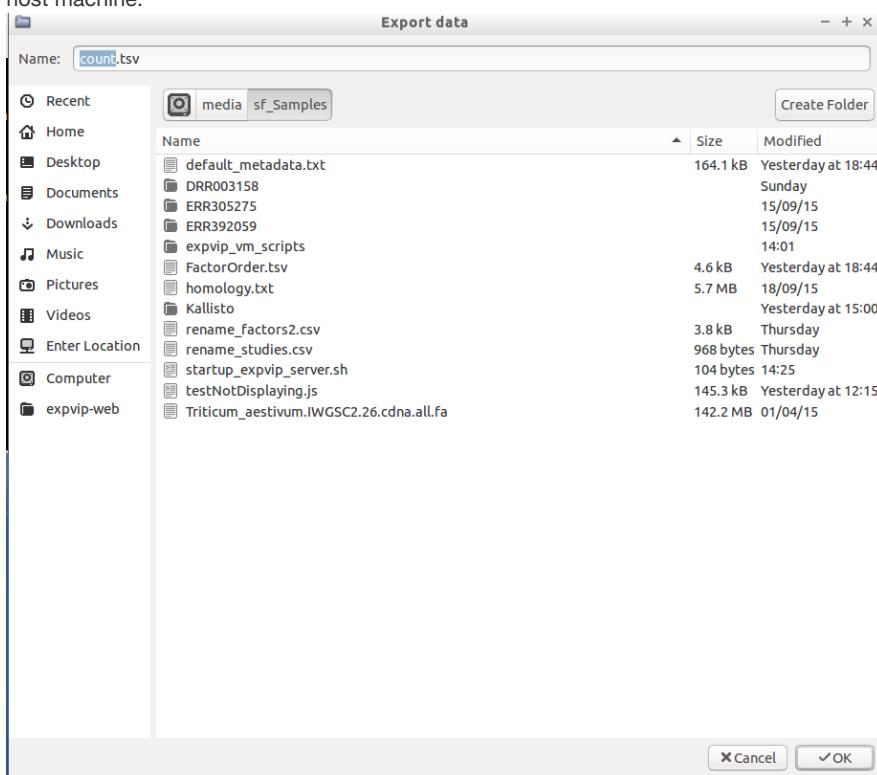
2. Execute it on the terminal



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 task

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

Abundance files

To use Sleuth, the abundance files from the `kallisto` runs can be grabbed 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 the [here](#).

Troubleshooting

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:

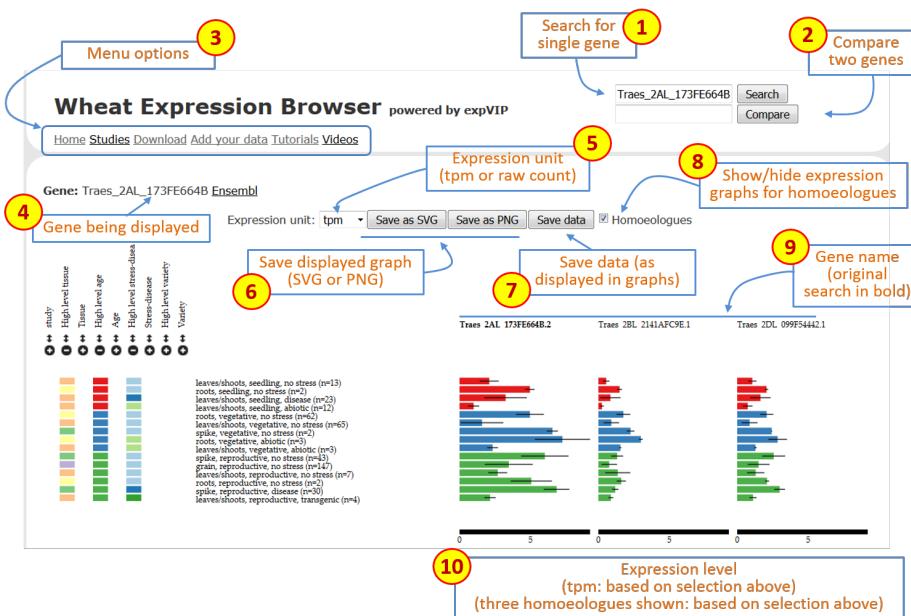


Figure 1: Overall description of features on Wheat Expression Browser

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).

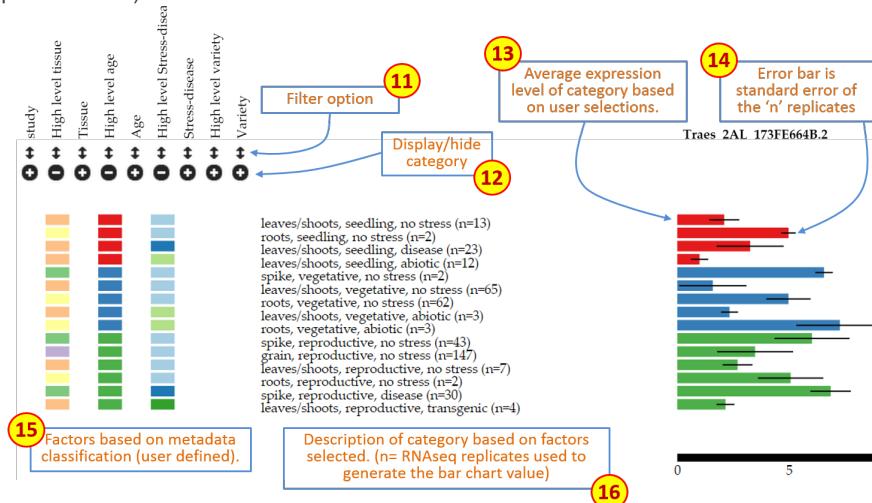


Figure 2: Overall description of features on Wheat Expression Browser (continued)

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 zero (i.e. $\log_2(<1)=0$).

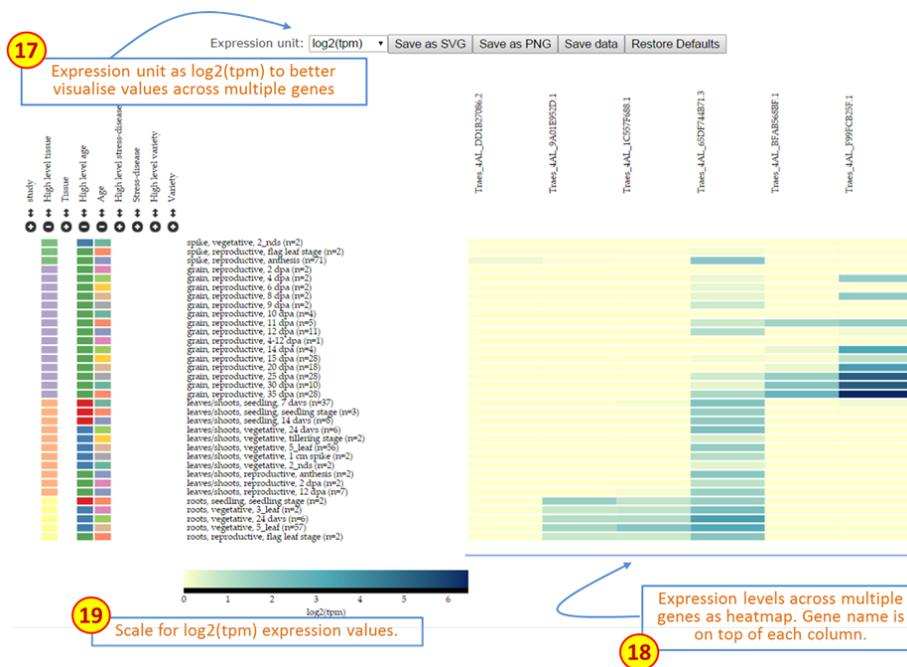


Figure 3: Description of features on Wheat Expression Browser using Multiple gene comparisons.

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).

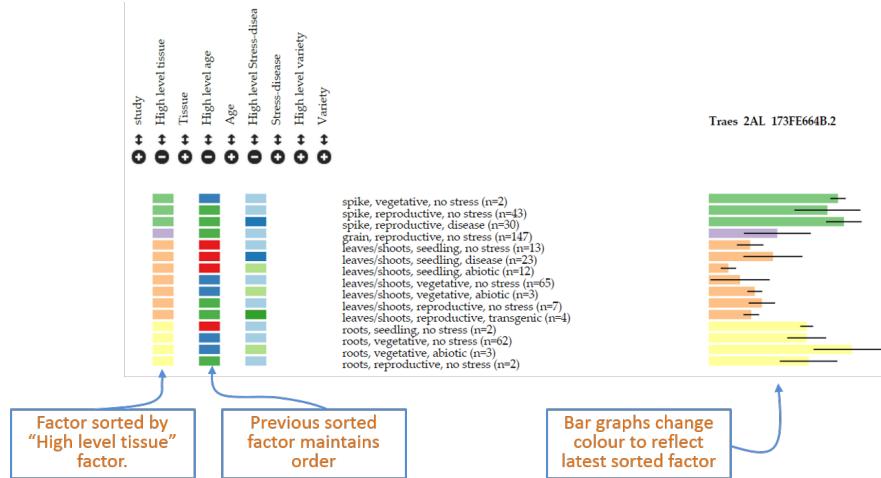


Figure 4: Example of new sorting of data based on clicking of rectangles within “high level tissue”.

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 .

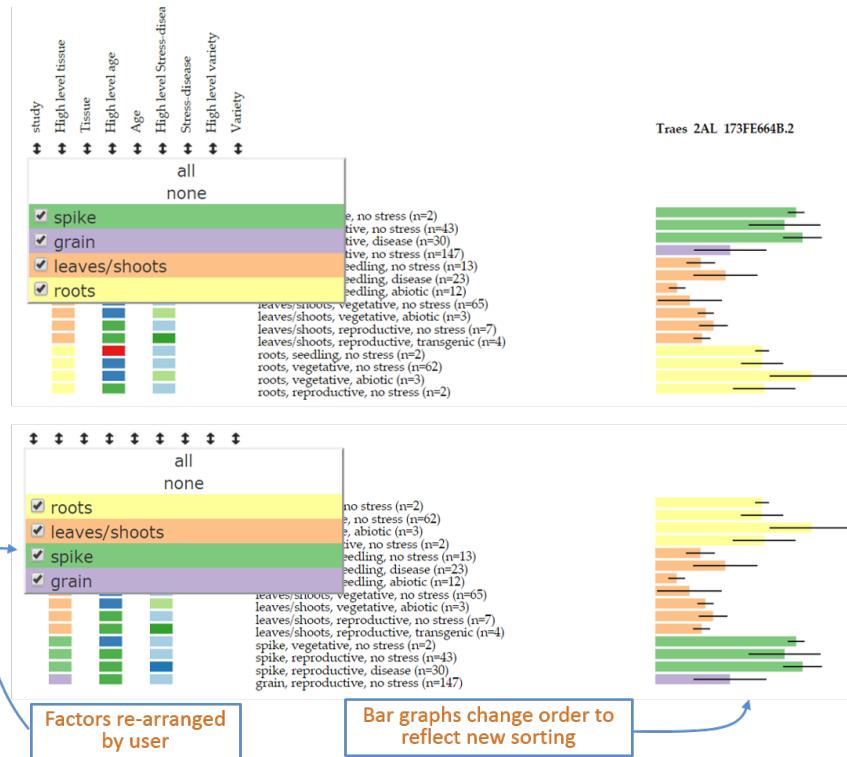


Figure 5: Example of sorting of data based on new user defined order within the filter pop-up window.

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.

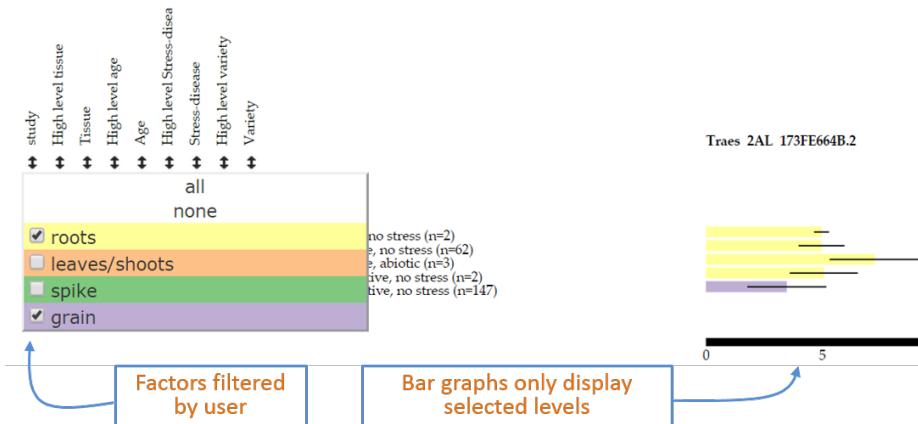
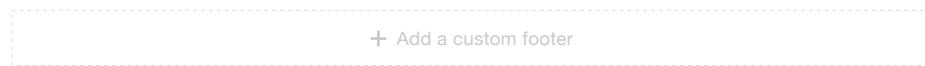


Figure 6: Example of filtering data based on user defined selection within the filter pop-up window.



Bibliography

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