Contents

Chapter 1

Introduction

It defines the objectives and the importance of the research. It focus on the the application of Next Generation Sequencing to molecular biology, wheat genetics and ultimately to breeding programs. It also mentions the current status of the wheat reference genome and other resources (genetic maps, markers) the need of tools to query them effectively.

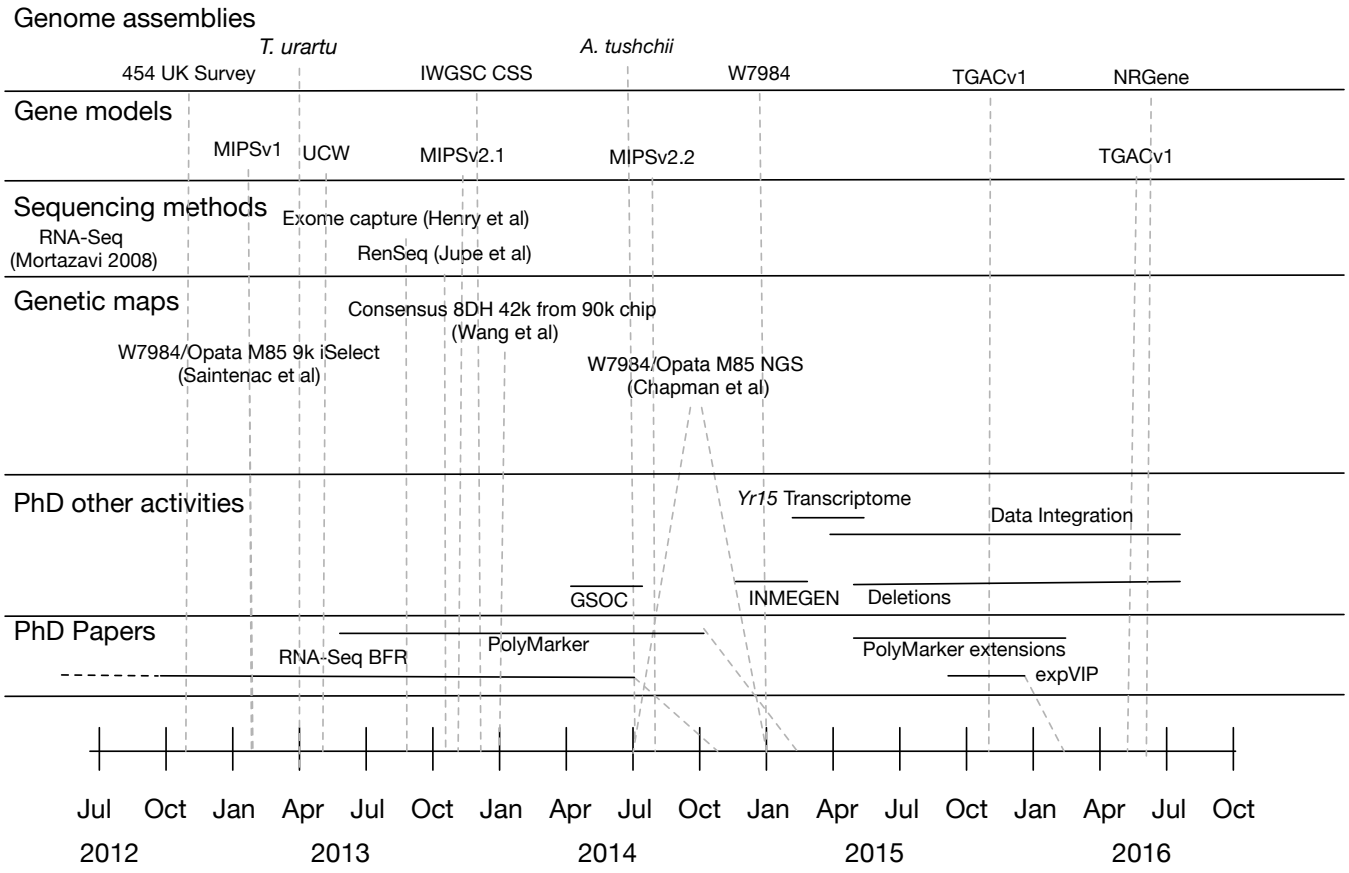


Figure 1.1: Timeline of the projects carried on during this PhD and the wheat resources that were released in the same period of time.

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 discuses 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 arouse, *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 paralogues.

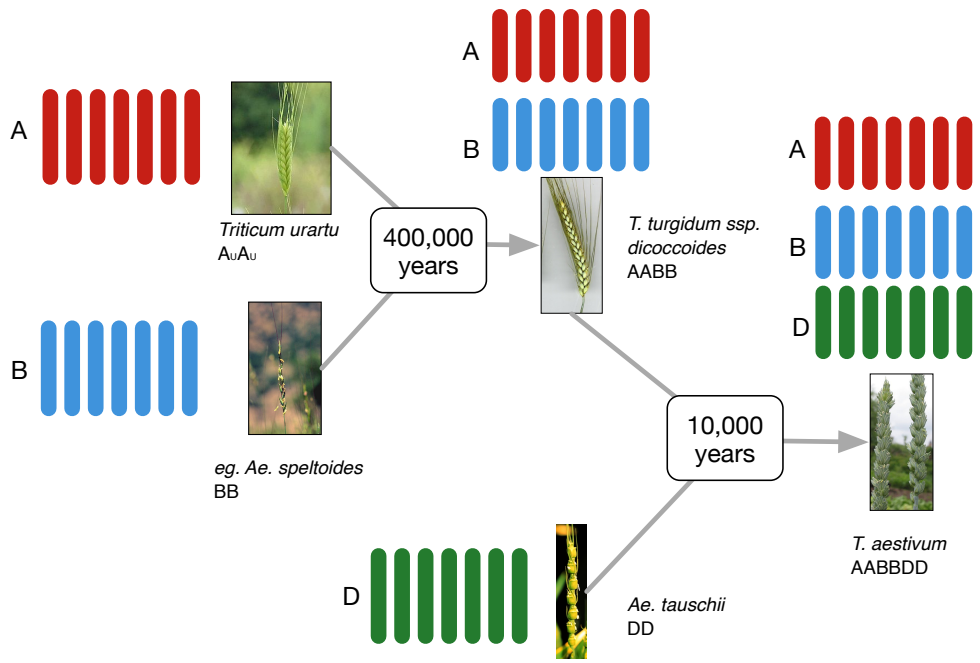


Figure 1.2: Hibridizations that lead to bread wheat *T. aestivum*.

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 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 Immunopresipitation is used to find relationships between proteins and DNA sequence. It is useful to find transcription factors and replication-related proteins.

Amplicon sequencing . Used primary 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 bellow:

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. The 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 use 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 gig abases 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 appear (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 yleds 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 marked 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-througput 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 enzimes 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 organisims 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 interns of accuracy looking for similarity between sequences. However, the execution time for both of them is prohibitive to run in massive databases. The algorithm execution time is O(mn), as it requires calculating a matrix of size 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 classifierEddy (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 experimentLiu 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/cufflinkns)

***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 populaiton, 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 whet genetics and genomics. MAS wheat, CeralsDB, Ensembl, etc.

1.8 Programming languages

Why Ruby and javascript? -Ruby -BioRuby -JavaScript -BioJS -Rails. -SQL

-lamda functions -functions/methods

-object orientation -hashes -design patterns -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 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' GAAGGTGACCAAGTTCATGCT 3'), which is used to distinguish between them (Figure 2.2a). The KASP mix contains complementing oligos to the HEX and FAM tail, which contain a dye that is only visible when the corresponding allele has amplified. The intensity of each dye is used to measure relative amplification of each allele. 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

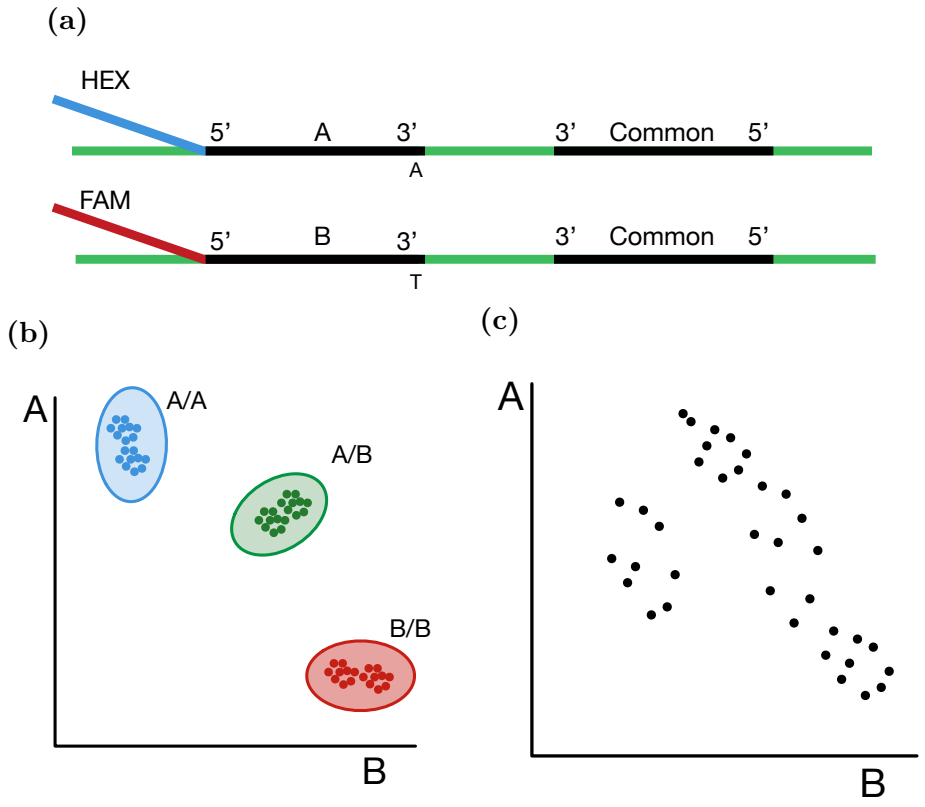


Figure 2.2: Kasp Assays (a) A KASP assay consists on three primers. Primers A and B are specific for certain allele and the HEX and FAM tails are added at the 5'-end on each primer. The common primer amplifies both possible products. The SNP is an A/T, the only difference between alleles. (b) Ideal KASP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

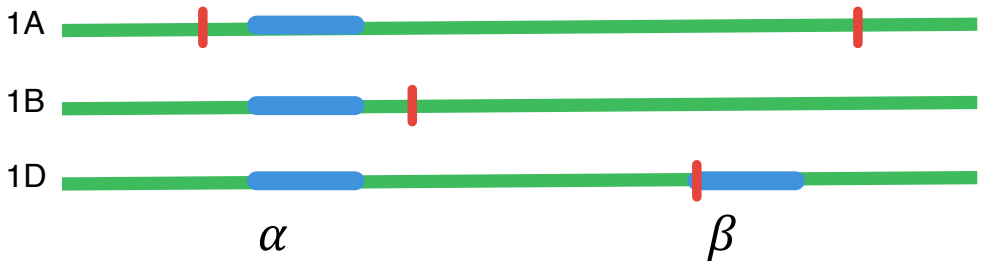


Figure 2.3: Primers selected randomly (blue lines) can bind to any of the three homoeologous regions if they fall on regions without variations between them (red vertical lines). The α primer doesn't contain any variation between chromosomes, hence it will bind to the chromosomes 1A, 1B and 1D. The β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

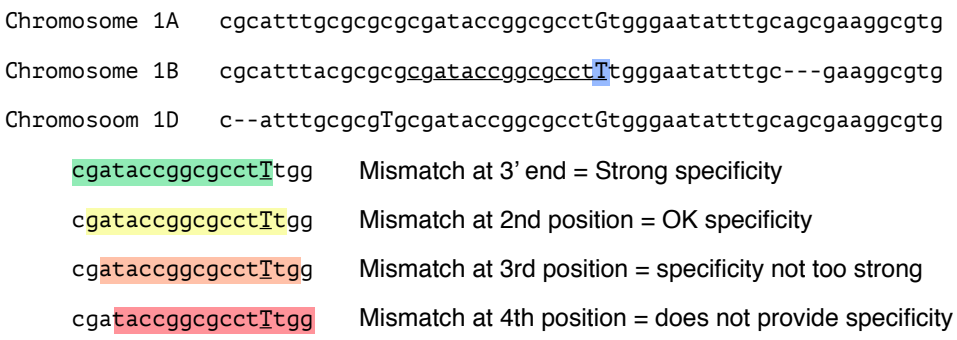


Figure 2.4: Several candidates to design a genome specific primer for chromosome 1B. The T highlighted in blue is a variation unique to the target chromosome. The closer the T providing specificity is to the 3' of the primer, the more specific it is.

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 specificitt 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 positionl no 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 constrains 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 this tools take a reference sequence and make some sort of index to speed up the search of the queried sequence 2.5a. 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 necesary to make a local alignment.

2. To put all the sequences in the appropriate context, a local alignment is done (Figure 2.5b). This is done by extracting all the hits to the target reference and using a program like `mafft` (Katoh and

Standley, 2013) or `clustal` (Higgins and Sharp, 1988). This 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 were 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).

If possible, expand and add diagram

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).

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

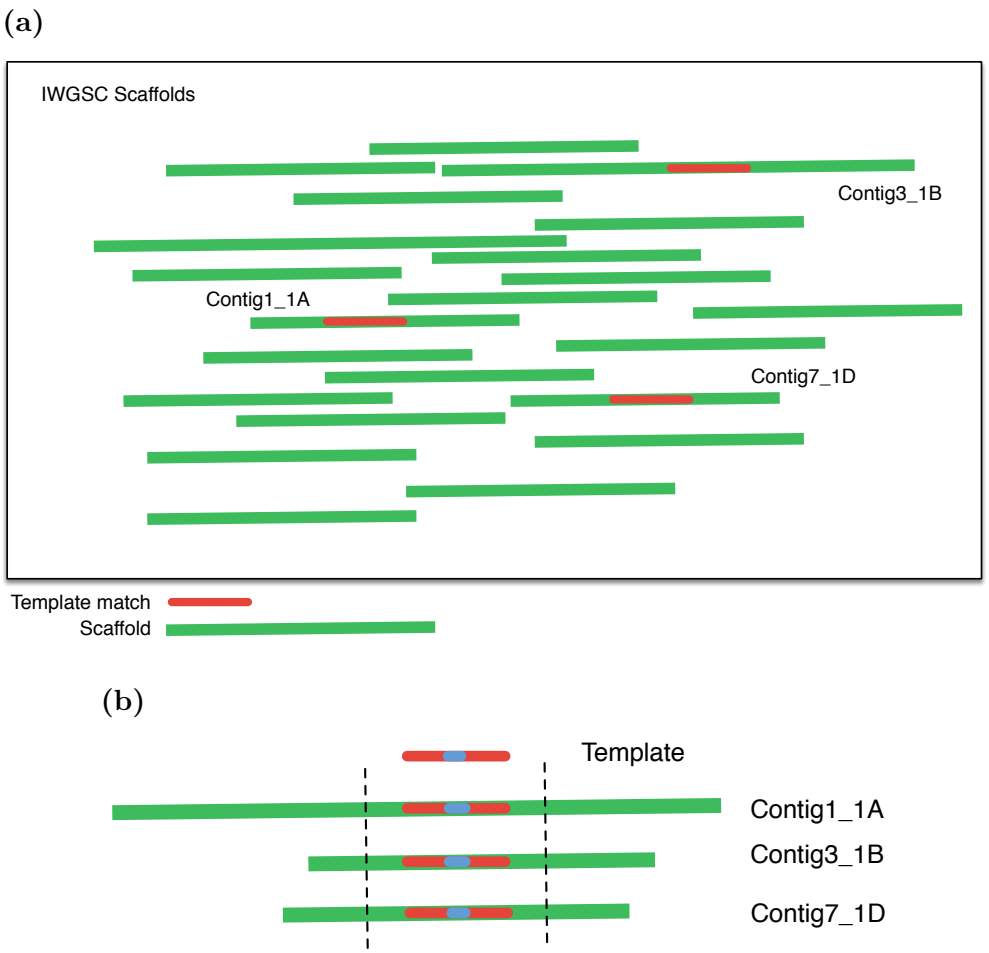


Figure 2.5: (a) Global search of templates in the reference contigs. (b) Selected regions around the SNP on every chromosome. The blue line represents the position of the SNP.

SNP and a base that is specific to the target genome (Figure 2.6). The pipeline is written as a Ruby script, using parsers and wrappers from BioRuby (Goto et al., 2010) and bio-samtools (Etherington et al., 2015; Ramirez-Gonzalez et al., 2012). The software is open source and released as a biogem (Bonnal et al., 2012), `bio-polyploid-tools`, the source code is available in: `https://github.com/TGAC/bioruby-polyploid-tools`.

The PolyMarker input consists on SNP list with: unique name for the marker, the target chromosome and the sequence for the marker. The alternative alleles are flanked by square brackets within the sequence. PolyMarker can take a list of several markers and design them in batch (Figure 2.7). A `FASTA` file is produced with all the template sequences, with the alternative alleles substituted by the IUAPC ambiguity codes (Cornish-Bowden, 1985). The flanking sequence surrounding the SNP is limited by default to 100bp to reduce the search time and avoid missing regions that diverge near the SNP, as when the variation is near an intron-exon junction.

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

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

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

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

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

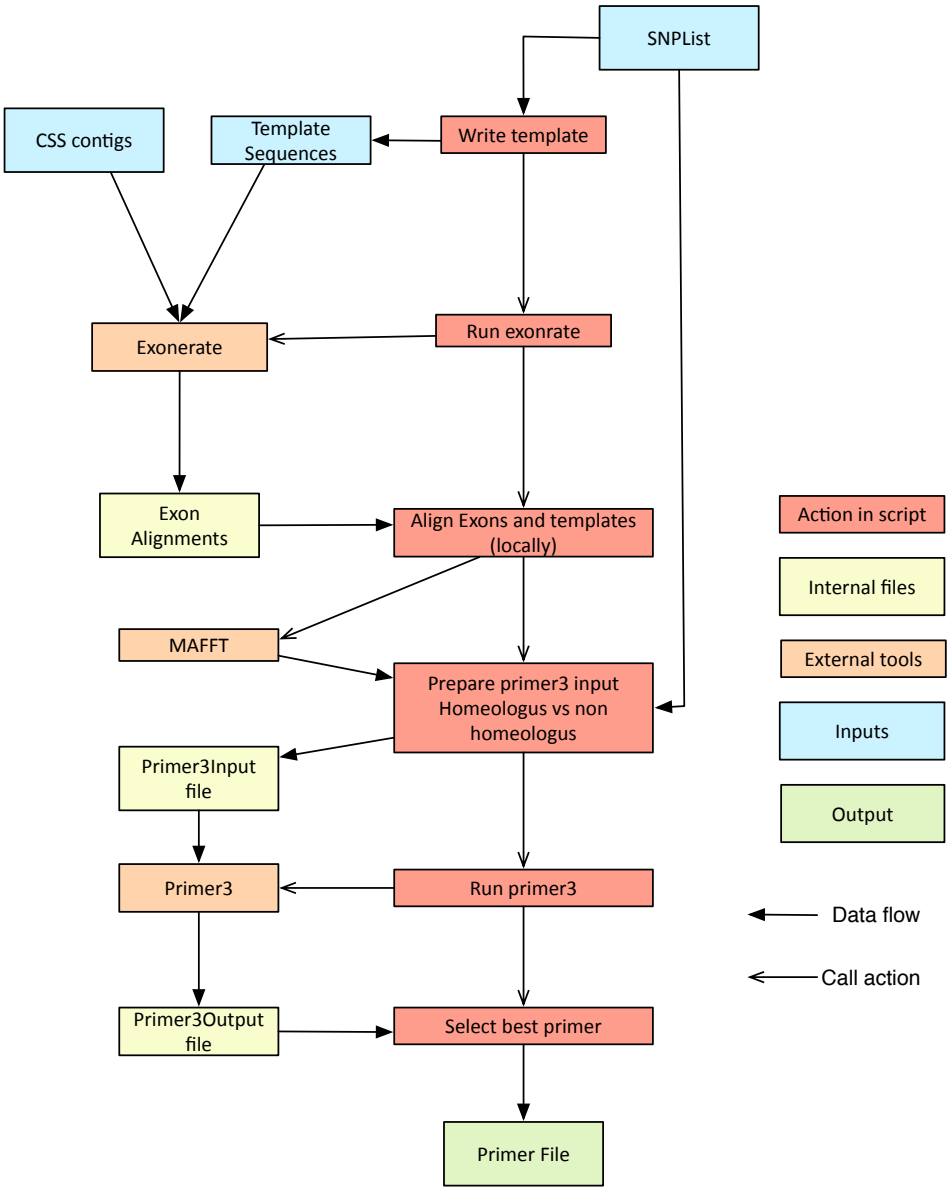


Figure 2.6: Steps and tools called by PolyMarker. The colour of the boxes represent: the step is an action inside the script(red); actions of the script(light red); temporary files(yellow); inputs(blue) and; outpus(green)

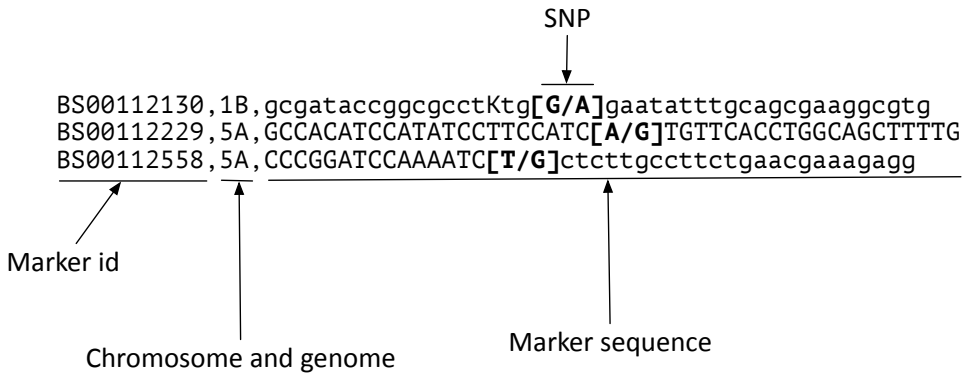


Figure 2.7: PolyMarker input. The alternative alleles are surrounded by brackets. The rest of the figures are based on BS00112130, renamed as SNP-1.

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

Figure 2.8: Sequence of flanking regions around the SNP. The indels produce a slight shift on the sequence.

paralogs. The coordinate of the SNP is calculated and 100bp on each flank are extracted by default, a reasonable product size for KASP assays. The flanking sequence may contain indels and the sequences don't align naturally (Figure 2.8). 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 know that the SNP comes from DNA, `affine:local` would be a better option as `exonerate` won't pay attention to the intron-exon junctions).

Each SNP marker is represented on the `BIO::PolyploidTools::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) that holds the SNP set and parses each output for all the called programs. The container for exonerate is `BIO:PolyploidTools::ExonContainer`. The hits with the SNP 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`

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

is to parse the `exonrate` output and add it to the corresponding SNP (Listing 2.1).

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

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

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

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

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

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

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

Figure 2.9: Local alignment on regions around the SNP detects indels.

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

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

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

To ensure that the indels between homoeologues don't produce spurious mismatches a local alignment is produced with `MAFFT` (Figure 2.9). The arguments used are the recommended in the manual for small number of sequences:

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

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

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

The class `BIO::PolyploidTools::SNP` has the method `aligned_sequences` which 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 2.10, 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 2.10, line 5).

2. Then each position in the mask is iterated base per base (Listing 2.10, line 7).

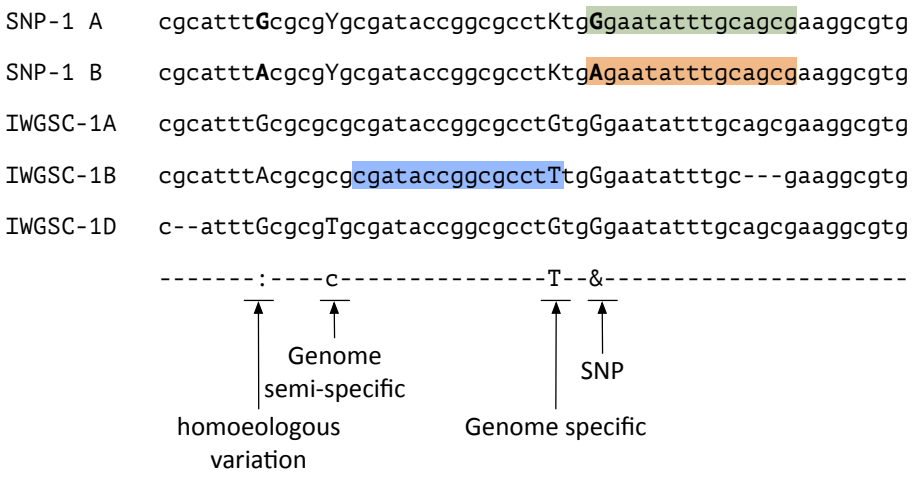


Figure 2.10: Alignment with mask and primer candidates. The green and light red boxes highlight the allele specific primers. The blue box highlights a genome specific primer.

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 polyploidy.

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

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

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

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

`PRIMER_PRODUCT_SIZE_RANGE=50-150.` 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 `--primer_3_preferences FILE`.

The input file for `primer3` also include the template sequences with an ID. To keep track of what kind of marker each position will produce the ID field has the name of the primer and the specificity of the starting position of the common primer. The starting position of the primers is forced with the options `SEQUENCE_FORCE_LEFT_END` and `SEQUENCE_FORCE_RIGHT_END` on the specific and 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 that calculates the mask of the alignment

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

The class `BIO::DB::Primer3::Primer3Record` is used to keep the details of all the primers generated by `primer3` for each template. In order to 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 2.10, 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 2.11). 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-homoeologus position with a genome-specific triplet (Figure 2.11a). However, sometimes the SNP is located in an homoeologous variation (Figure 2.11b), 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 2.11c), 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

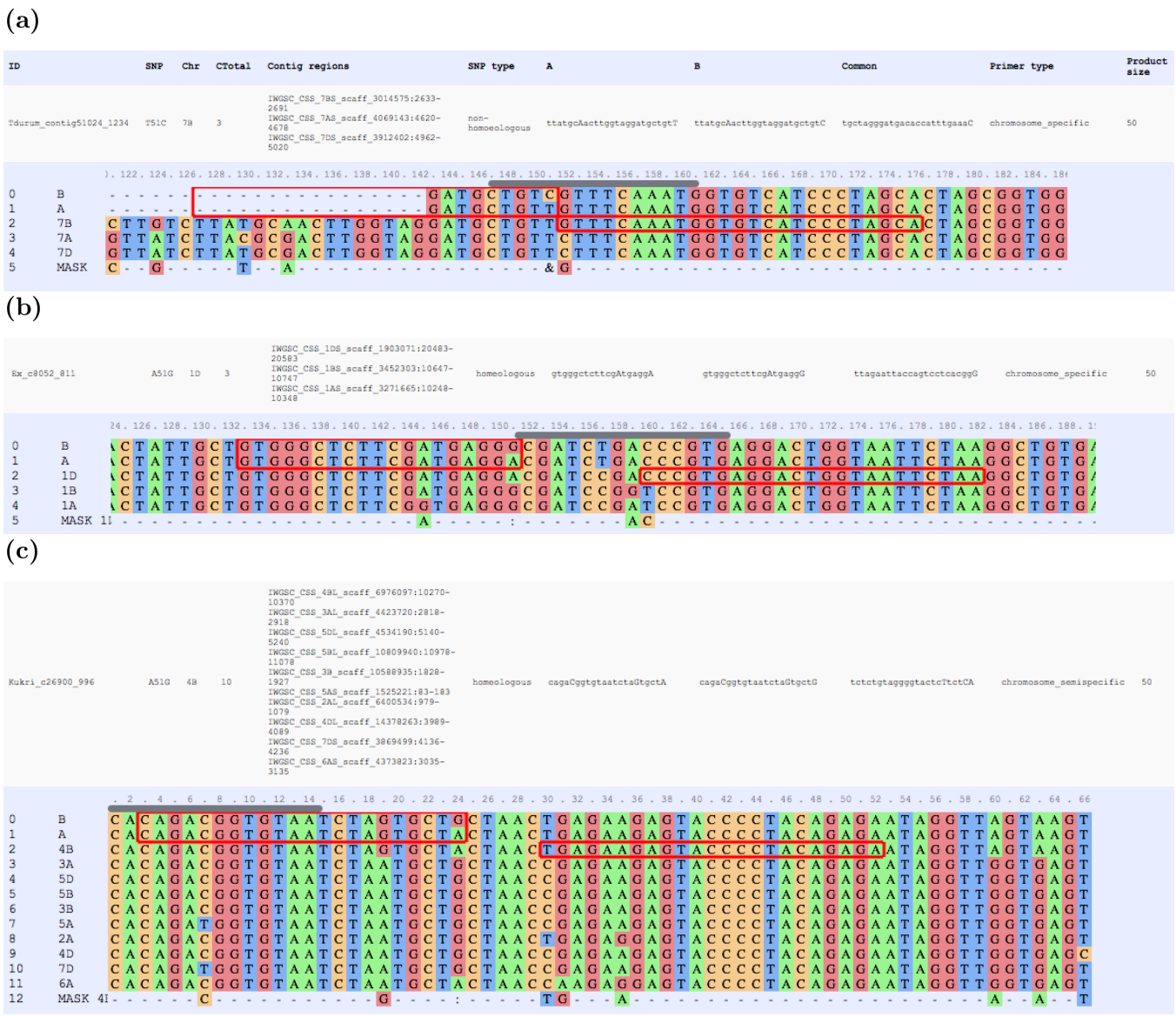


Figure 2.11: Example outputs from the PolyMarker website. (a) The primer triplet is genome specific. The original marker sequence had the SNP near the begining of the template, but PolyMarker used the genomic reference to complement the sequence. (b) An specific primer, but the SNP is located on the 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 `exonerate` (Slater and Birney, 2005) to the CSS scaffolds. Of those, 86.1% have an specific or semi-specific assay (Table 2.2. This set of primers is also available in CerealsDB and it provides a valuable resource to groups that want to genotype using a subset of SNPs in the array, without the need to run the 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-specifc	167,018	132,145	48.52%
Specific	139,202	92,487	37.58%
Not designed	3,116	5,852	1.45%
Total	329,525	287,000	616,525

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 asses 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) where 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 where 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 validataed 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 2.12a, b; reverse primer). On lines without the targeted deletion, the amplification corresponds to an hetrerozygous assay with equal signal for both the A and the B allele. (Figure 2.12c). 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 2.12d).

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.

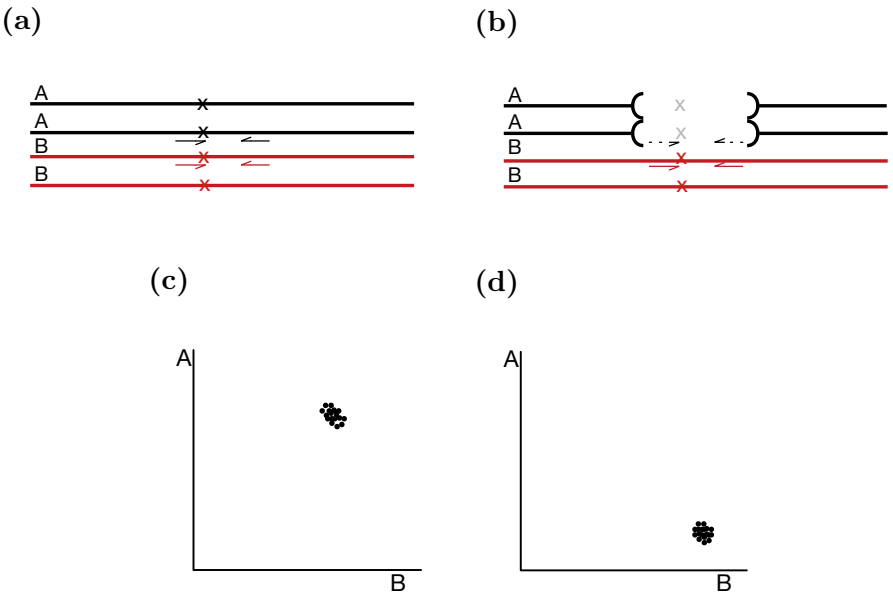


Figure 2.12: KASP assays to validate homozygous deletions. (a) Primer positions for wildtype. Red and black indicate the A and B genome respectively. Primers are indicated by arrows with the target homoeologous SNP marked by an "X"(b) Primer positions on homozygous deletion on M_4 (c) Heterozygous amplification 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-specifc primers

```
kasp_container.scores[:chromosome_specific] = 0
kasp_container.scores[:chromosome_semispecific] = 1000
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

```
arm_selection_functions[:pst130] = lambda do |contig_name|
  return "PST130"
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	C	Result
5BS_2297308_Cadenza0423_12664_C12664T	-	5B	4.551	X	X	-	X	X	X	X	X	X	X	-	X	Y	Y	Y	Y	HOM Mutation
5BL_10812849_Cadenza0423_5664_G5664T	-	5B	38.769	X	X	-	X	X	X	X	X	X	X	-	X	Y	Y	Y	Y	HOM Mutation
5BL_10825062_Cadenza0423_7917_G7917A	-	5B	38.769	X	X	-	X	X	X	X	X	X	X	-	X	Y	Y	Y	Y	HOM Mutation
IWGSC_CSS_5BL_scaff_10847976:27068-27231	+	5B	38.769	X	X	-	X	X	X	X	X	X	X	-	X	H	H	H	H	Hom Deletion
IWGSC_CSS_5BL_scaff_10847976:28118-28674	+	5B	38.769	X	X	-	X	X	X	X	X	X	X	-	X	H	H	H	H	Hom Deletion
IWGSC_CSS_5BL_scaff_10865441:15863-15946	+	5B	38.769	X	X	-	X	X	X	X	X	X	X	-	X	H	H	H	H	Hom Deletion
5BL_10837222_Cadenza0423_4616_G4616A	-	5B	39.905	X	X	-	X	X	X	X	X	X	X	-	X	Y	Y	Y	Y	HOM Mutation
5BL_10891320_Cadenza0423_18847_C18847T	-	5B	45.594	Y	Y	-	Y	H	X	X	Y	H	Y	-	H	Y	Y	Y	Y	HET Mutation

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

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

2.4 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 tame, the IUAPC ambiguity codes (Cornish-Bowden, 1985) were useful in the development of PolyMarker. With an ambiguity code, the template for the search can contain the SNP. Also, the codes were useful when representing all the observed bases on each coordinate in the local alignment.

The ideas behind PolyMarker had been taken by other projects like the scripts described in Ma et al. (2015) and the corresponding web interface, GSP (Wang et al., 2016). Briefly, GSP does a blast search to find all the homoeologus regions and provides a diagram with the bases that are genome specific. It then allows the user to select a primer pair according to the constrains for 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 require a SNP sequence, does the alignment and selects primers with a genome specific anchor. As PolyMarker, it produces a local alignment with the genome-specific bases (Curry et al., 2016). On personal communications in conferences

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-polyploid-tools`.

Chapter 3

Genetic map of *Yr15* with RNA-Seq

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 diccocides*. 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.

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 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).

Bulk Segregant Analysis (BSA) consists on pooling the DNA of individuals with contrasting phenotypes (Michelmore et al., 1991) on a

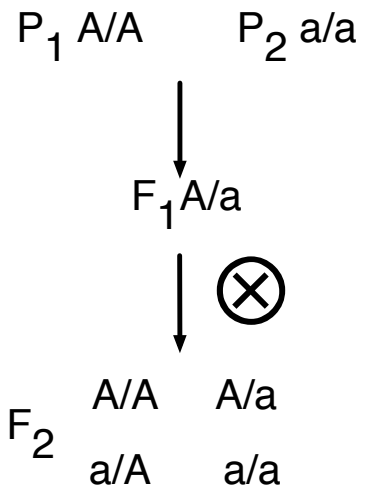


Figure 3.1: The cross of two homozygous parents, P_1 and P_2, with a dominant and a recessive allele of a gene produce an heterozygous F_1. The F_1 crossed with itself produce a segregating F_2 population with a 1:2:1 ratio (A/A:A/a:a/a). The upper and lower cases represent dominant and recessive alleles

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 resquencing (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 homoeologues 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 3.9.3 and the results on the F_2 population are discussed in Section 3.4.

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 paralogue 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 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 3.5. For a longer description of resources available for wheat see Section 1.7.

To do: section talking about genetic map.

Finally, the best candidate SNPs where 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: Microsatellites vs SNP markers.

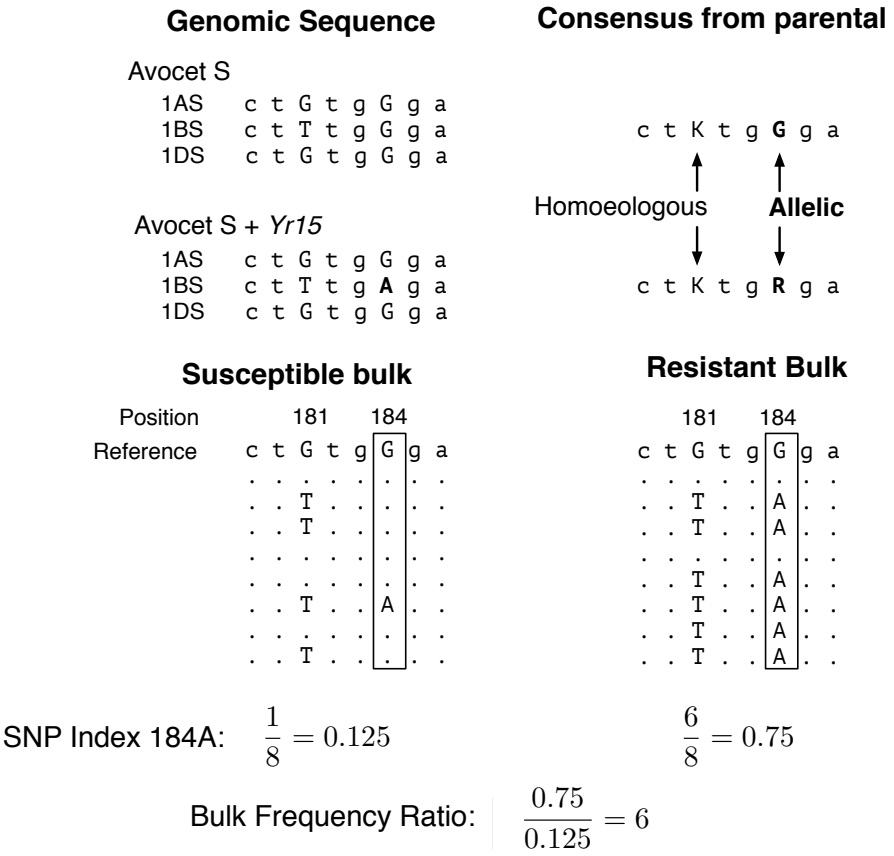


Figure 3.2: Illustration of a non-informative homoeologous SNP (G181T) present in both parental lines, and an informative allelic SNP (G184A), only present in the resistant progenitor Avocet S + Yr15. The consensus sequences from the parental genotypes include this information in the form of ambiguity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

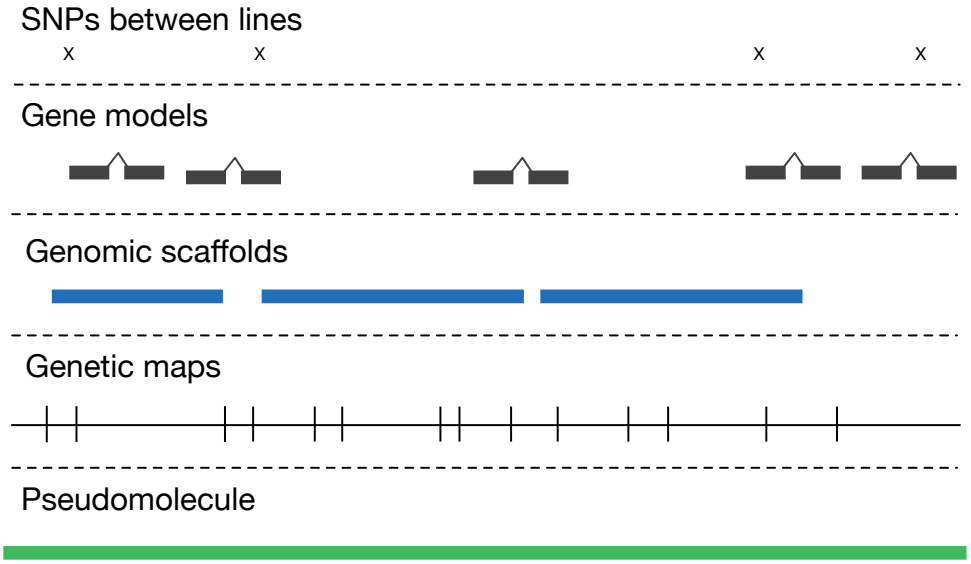


Figure 3.3: Layers of information to do *In Silico* mapping. SNPs are called from gene models. The genes and markers from genetic maps are aligned to scaffolds. The order of the markers in a genetic map can be used to sort the scaffolds.

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 succeptible to yellow rust, hence the resistance is coffered by the *Yr15* locus. F_2 seeds from tree independent F_1 plants where sown and tissue was collected, before the fungal inoculation to avoid the effect of the response on the gene expression. The plants were challenged at the three leaf stage as it is know that *Yr15* confers resistance in seedlings (Gerechter-Amitai et al., 1989). The expected segregation on an F_2 population is 3:1 (resistant:susceptible), since *Yr15* is a dominant gene. From the 232 plants in the F_2 population that germinated, 187 were resistant and 45 were susceptible, which deviates slightly from the expected ratio ($\chi^2 = 0.049$). Segregation distortion has been shown for the same *Yr15* donnor (Randhawa et al., 2009), however the decresed number of succeptible plants can be explained by escapes in the virulence essays (i.e. plants scored as resistant without the *Yr15* locus). For this study we extracted DNA from individual plants in the F_2 population and we bulked RNA on 6 different bulks: 3 resistant and, 3 succeptible (Figure 3.4c).

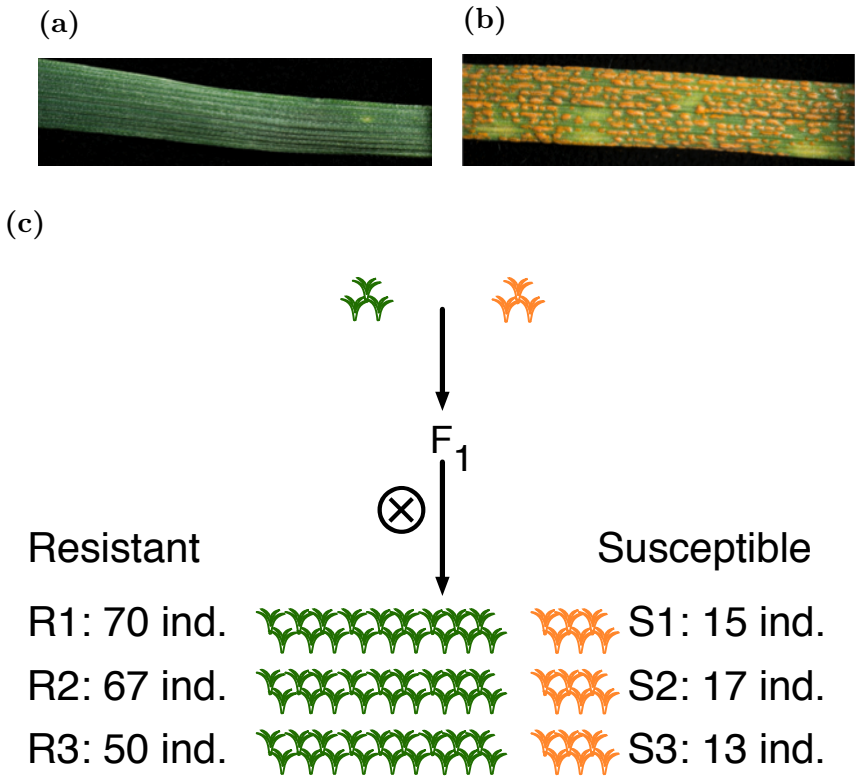


Figure 3.4: Response of (a) Avocet + *Yr15* and (b) Avocet when inoculated with *Puccinia striiformis* f. sp. *tritici* at the three leaf stage. (c) The phenotype of the F_2 population was used to produce 6 bulks, 3 resistant and 2 susceptible. The RNA was pooled in bulks accordingly. Adapted from (Ramirez-Gonzalez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

3.1 Mapping population

3.2 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 sequencencing 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%.

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

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

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 not release yet, hence gene models where used. All the samples where 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 have 22,107 and 36,808 genes, on the Unigenes and the UCW gene set respectivley, 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% respetively 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 susceptible (S1 + S2 + S3) and resistant bulks (R1 + R2 + R3). The merged samples increased the percentage of genes with coverage over 20x to 44% and 50% in the resistant and susceptible bulks (Table 3.2), which is close to the coverage from the progenitors.

3.3 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

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

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

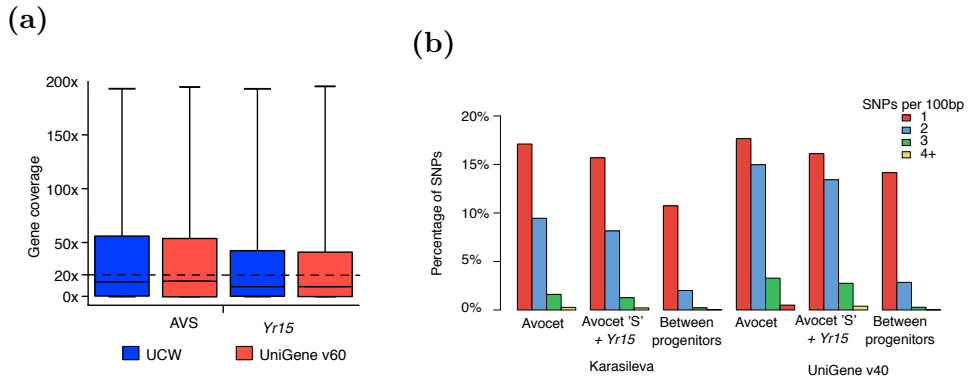


Figure 3.5: (a) Box plot distribution of the gene coverage of the parent reads (AVS and *Yr15*) across the UCW (blue) and the UniGene (red) gene models. The dashed line represents the 209 minimum coverage required for SNP calling. The full line represents the average coverage across all gene models. (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)

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.

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 were 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).

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

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

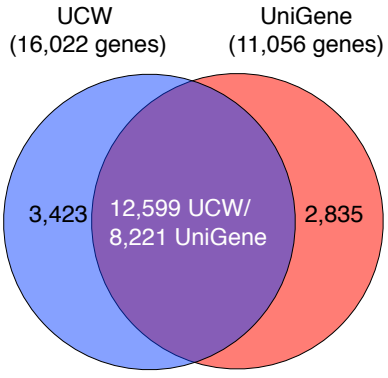


Figure 3.6: Gene models with putative SNPs in common between the UCW and UniGenes reference. The intersection represnts the genes that are common in both sets. Adapted from Ramirez-Gonzalez et al. (2015c)

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

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 survey 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.

3.4 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

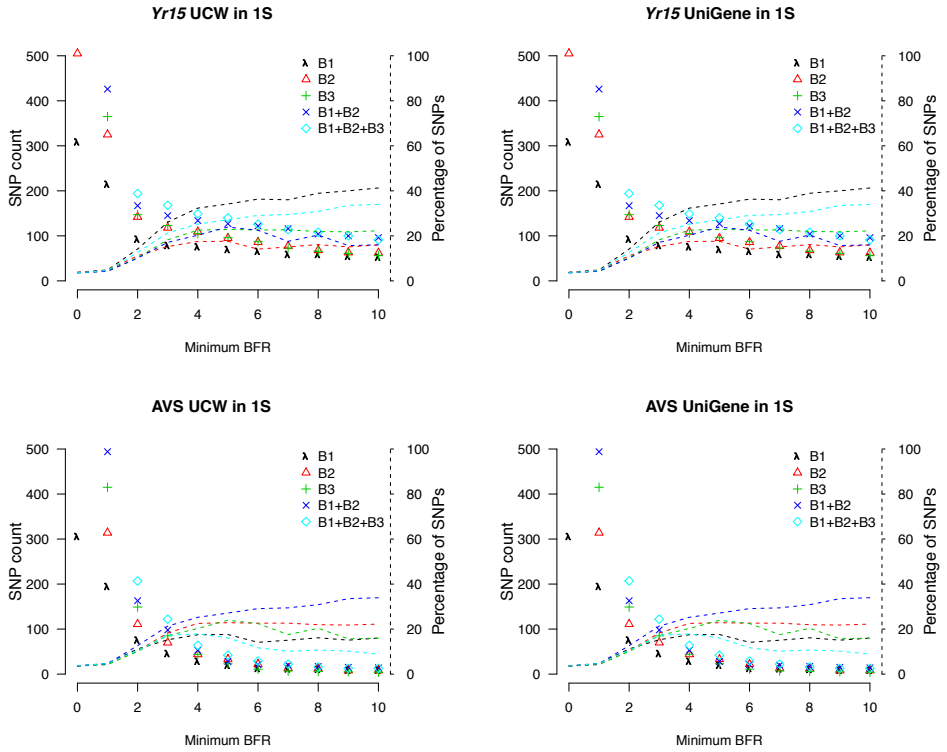


Figure 3.7: Effect of BFR threshold on the number of SNPs across the short arm of chromosome group 1. Figure previously published in Ramirez-Gonzalez et al. (2015c).

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 3.9.3), which has a value that increases as the *Yr15* allele is observed more times relatively to the AVS allele.

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

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

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

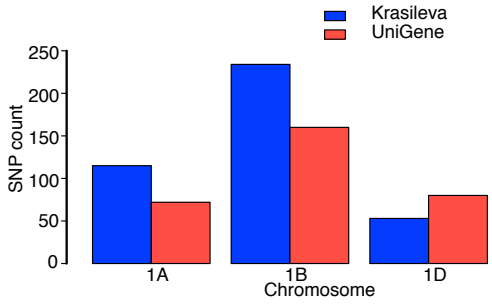


Figure 3.8: Location of SNPs with $BFR > 6$ according to the best alignment of the UniGene (red) and UCW (blue) gene models to the flow-sorted group 1 chromosomes from the Chinese Spring Survey sequence (CSS) (Mayer et al., 2014). Figure adapted from Ramirez-Gonzalez et al. (2015c).

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

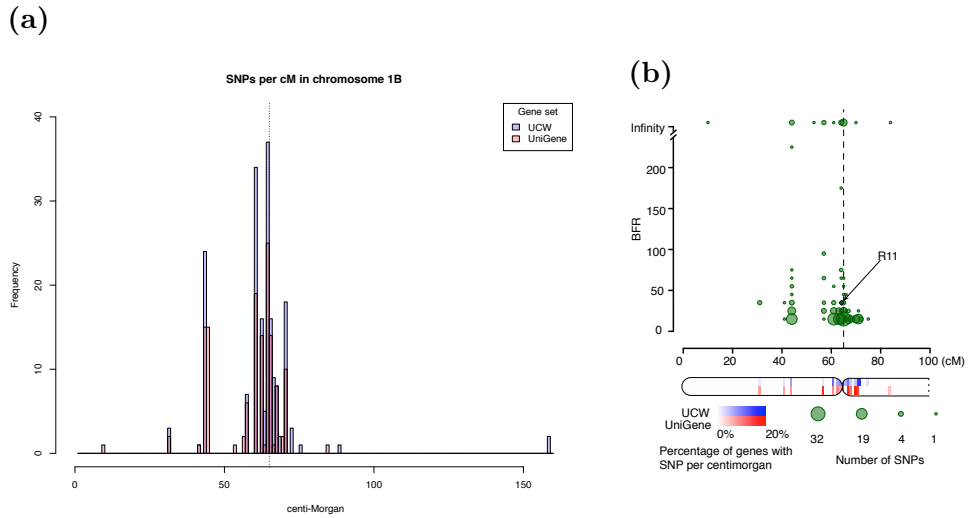


Figure 3.9: (a) Number of SNPs with $BFR > 6$ per cM in chromosome 1B. (b) BFRs of mapped genes with SNPs on chromosome 1B. The area of the circle represents the number of SNPs clustered by location (windows size: 10 cM) and BFR (window size: 5cM). R11 is the only marker near the *Yr15* locus that had 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.

3.6 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. diccocoides* 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

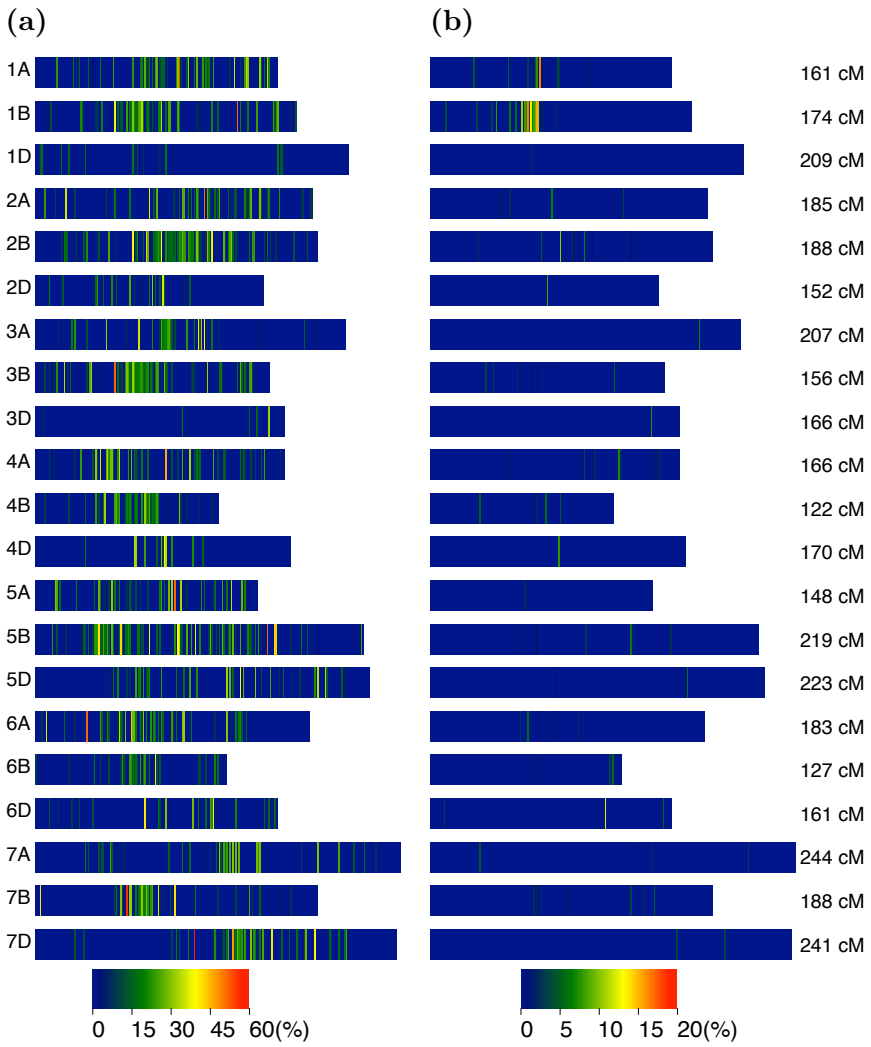


Figure 3.10: Genetic location of genes with SNPs between AVS and Yr15. The colour scale indicates the percentage of genes with SNPs per centi-Morgan (cM) across the 21 wheat chromosomes. The location of the genes was determined by the best alignment to the CSS scaffolds, and the location of these was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

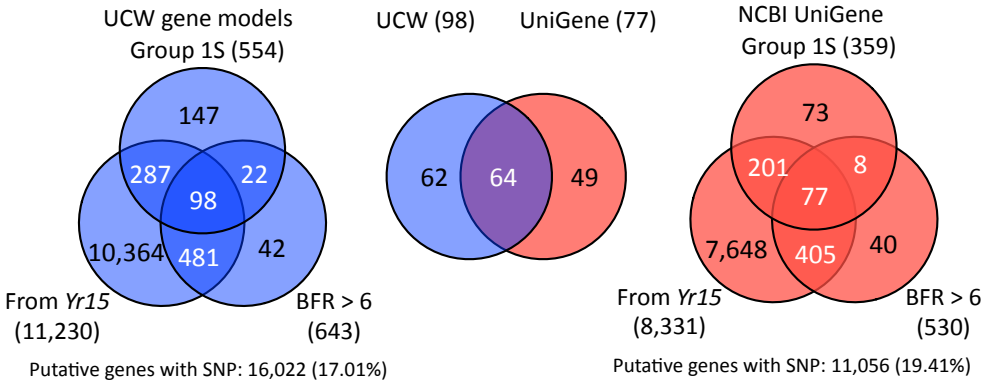


Figure 3.11: Selection criteria for marker design. Venn diagrams based on the three selection criteria (SNP in the short arm of chromosome group 1; SNP has a $BFR > 6$; and SNP is from the *Yr15* parent) for the UCW (blue) and UniGene (red) gene models. The centre diagram shows the intersection between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

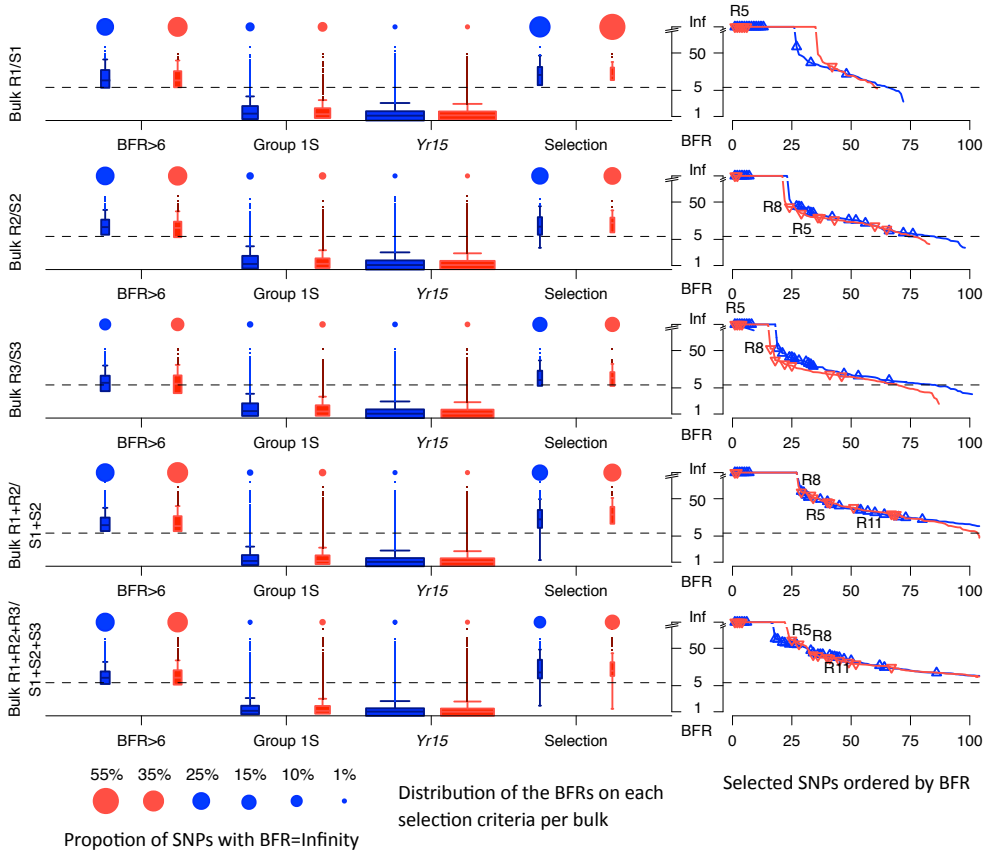


Figure 3.12: Bulk frequency ratio (BFR) of selected SNPs across the individual bulks and in silico mixes (UCW, red; UniGene, blue). The dotted line represents the BFR threshold of 6 (logarithmic scale). Left: Distribution of the BFRs for each selection criteria and the selected SNPs for validation. The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

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

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

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

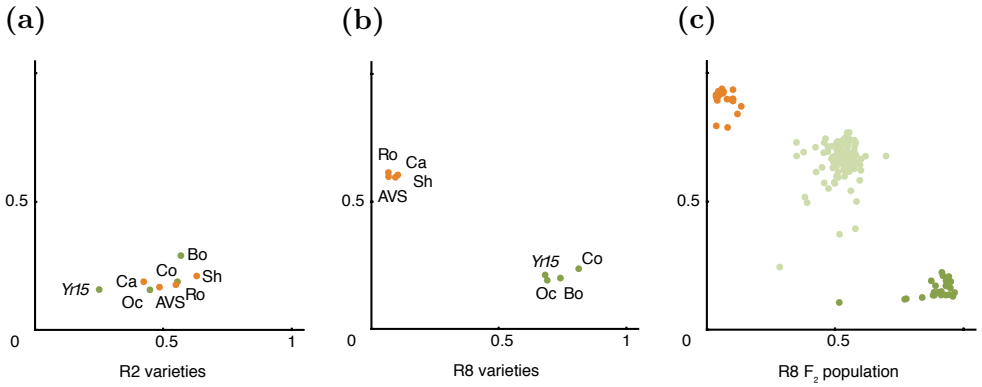


Figure 3.13: KASP output from the wheat variety panel with (Ochre, Boston, Cortez) and without (Robigus, Cadenza and Shamrock) *Yr15*. Marker R2 (a) is monomorphic while R8 (b) is polymorphic between varieties know to carry the gene. Marker R8 results for the F2 population (c) showing three distinct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

3.7 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 design as specific, 9 as semi-specific to chormosome 1BS, and 9 were not specific because there was no information for the homoeolouges 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 2.10; 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. diccocoides* in their pedigree (Shamrock and Robigus), as

Table 3.9: Primer details for the markers to validate.

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

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. diccocoides* instead of *Yr15*. On the test panel, 28 (80%) SNPs were polymorphic across the parents and three of them where diagnostic to *yr15* (R5, R8, R33). From the five homoeologous SNPs, three of them were monomorphic and 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 3.1, Figure 3.13c) and a genetic map was produced (Section 3.8).

3.8 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 3.6) for selecting candidate SNPs was effective. Then, the complete F_2 population was assessed with:

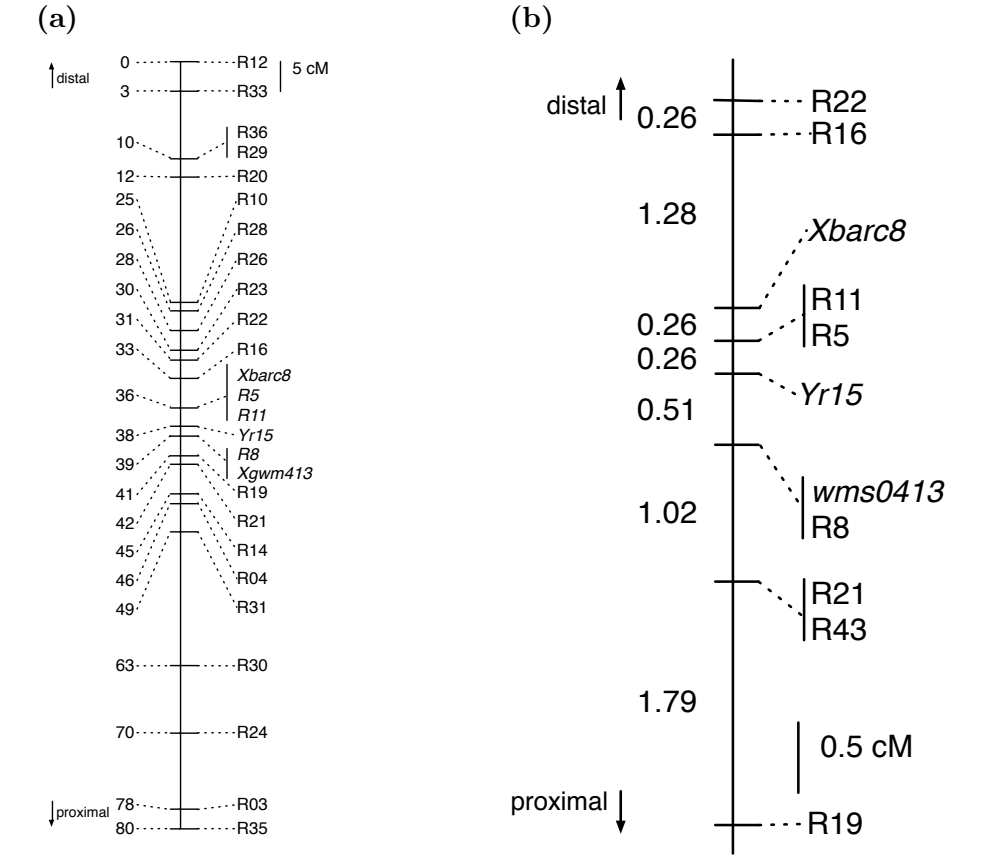
- the seven markers that were most linked to *Yr15*, including two of the diagnostic markers from the variety panel (R5 and R8),
- The flanking SSR microsatellite markers used by UK breeders for germoplasm selection (Xbarc8 and Xgwm413).
- A marker based on barley-wheat synteny (R43) which met the selection 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 SRRs, the *Yr15* locus was mapped to an interval of 0.77cM, with R8/xgwm413 0.26cM distal, and R5/R11 0.77cM proximal from *Yr15* (Figure 3.14b,c).

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



(c)

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

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

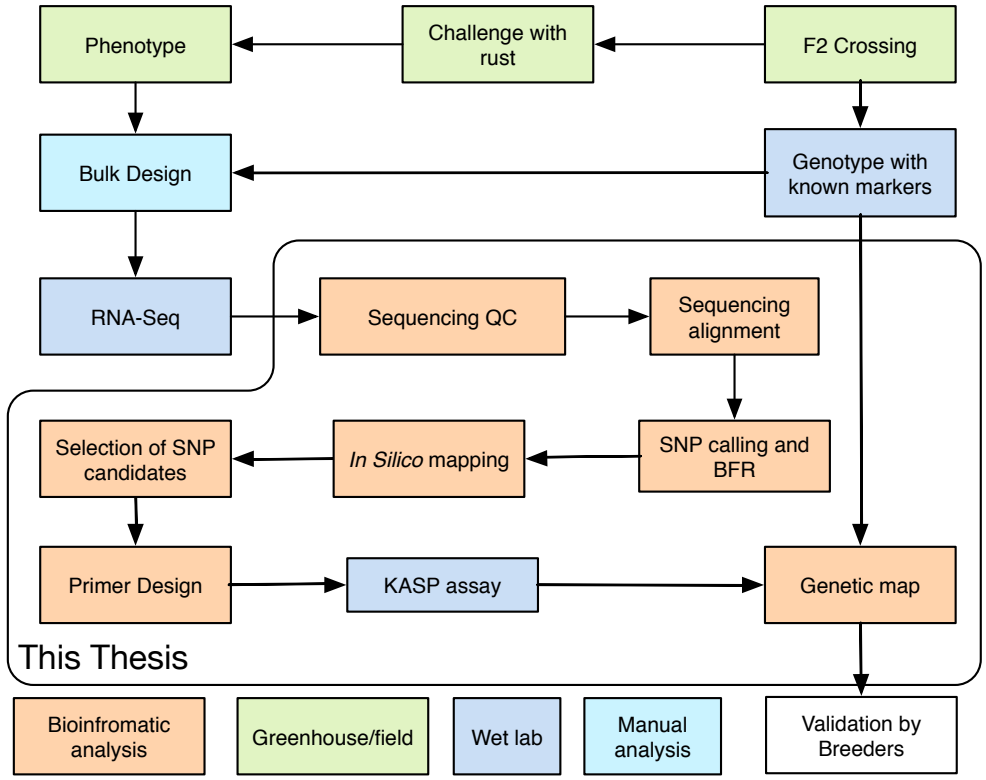


Figure 3.15: Steps used to go from the F_2 population to the genetic map.

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

3.9 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.

3.9.1 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 where left compressed and in chunks of 40,000, as teh 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).

3.9.2 Alignment reads to gene models

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

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

3.9.3 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 `minumum_ratio_for_iuap_consensus` the base is added to the set of possible bases (Cornish-Bowden, 1985) If there is no coverage at a certain position, the reference base is used, and set as lowercase. If the set of called bases is not empty, the ambiguity code for the observed bases is called, and set as upper case (Listing 3.1). The minimum ratio was done on 0.2 (20%), that allows for calling for a consensus even when more than one homoeologue is mapping to the same reference.

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

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

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

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

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

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

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

To calculate BFRs the class `Bio::BFRTools::Container` was implemented to contain all the `BIO:DB:Sam` objects corresponding to the progenitors and the bulks. The class `Bio::BFRTools::BFRRegion` was implemented to contain the ratios and consensus sequences of each region. The method `bfr` uses the calculated SNP-Indices on every position, from the point of view of both progenitors (lines 15-16: Listing 3.4, and in the case of lack of coverage the value is set to 0 or `Infinity` (lines 8-13), depending on the progenitor where the base is not called at all. Using this design 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: S1R1, Bulk 2: S2R2 and Bulk 3: S3R3); 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
```

3.9.4 *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`

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

Then, the UniGenes and the UCW gene models were also aligned with BLAT to the scaffolds that were located in the genetic map. The class `Bio::Blat::Report::Hit` was extended to calculate how many bases are covered in the alignment and the percentage of covered bases in both, the target and query sequences (Listing 3.6). Only the genes that align over 60% of covered bases with an identity of at least 90% were considered. This removes spurious mappings from repetitive regions while retaining 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 from `Bio::Blat::Report::Hit` to improve the filtering of spurious alignments.

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

3.9.5 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 standardFAMor HEX compatible tails (FAM tail: 50 GAAGGTGACCAAGTTCATGCT 30; HEX tail: 50 GAAGGTCGGAG TCAACGGATT30) with the target SNP at the 30 end. Primer mix was set up as recommended by LGC [46 lL dH2O, 30 lL common primer (100 lM) and 12 lL of each tailed primer (100 lM)] (LGC Genomics, 2014) Assays were tested in 384-well format and set up as 4-lL reactions [2-lL template (1020 ng of DNA), 1.944 lL of V4 29 Kaspar mix and 0.056 lL 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 C10 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 detected at ambient temperature. If the signature genotyping clusters had not formed after the initial amplification, additional amplification cycles (usually 510) were conducted, and the samples

were read again. Data analysis was performed manually using Kluster-caller software (version 2.22.0.5; LGC Hoddesdon, UK).", as described in Ramirez-Gonzalez et al. (2015c).

3.9.6 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..

3.10 Discussion

Resequencing the $\sim$ 17Gbp genome of hexaploid wheat is costly and approaches to reduce the required sequenced volume to effectively call for SNPs had been evolving since the conception of this project. 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 3.2). 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 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 aditional 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 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 where 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 3.5). 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 3.3). 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 3.6). 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 3.8). 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 use to repet a similar analysis, but sequencing with either exome capture or RenSeq.

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

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

> The markers R11, R5 and R8 were testedacross 122 doubled haploid (DH) lines. These DH lines were derived from

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

Figure 3.16: 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 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

This is 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

Gene expression (expVIP)

4.1 Expression experiments (Introduction)

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

4.2 Database design

Description of how the database was designed and the flexibility given by having the factors and units as variables

4.3 Analysis pipeline

Implementation of the pipeline, from running kallisto to load the data in the database

4.4 Graphical interface

How the expression can be displayed filtered, and sorted

4.5 Conclusions

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.

Chapter 5

Conclusions 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.

Appendix A

Supplemental tables

A.1 PolyMarker supplemental tables.

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

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

IWGSC contig	Line	Pos	WT	Mut	Predicted	M_4	Primer 1 (Cadenza)	Primer 2 (mutant)	Common Primer
IWGSC_CSS_2BL_scaff_7972799	Cadenza1469	8995	C	T	het	hom	gTgCtcctcGgcatccttC	gTgCtcctcGgcatccttT	gatccgGgcaaactacgTG
IWGSC_CSS_2DL_scaff_9832343	Cadenza1469	3262	G	A	het	het	TtgtctaAcagcacCGcagG	TtgtctaAcagcacCGcagA	agatctcggtcagcctttcT
IWGSC_CSS_2DS_scaff_5327939	Cadenza1469	3889	G	A	het	het	ttttTgccttatgtgactctagtaC	ttttTgccttatgtgactctagtaT	gaggccatcacagatagcG
IWGSC_CSS_3B_scaff_10395219	Cadenza1469	1292	G	A	hom	—	aggtgcttgtgcttgctgG	aggtgcttgtgcttgctgA	cctcttctgggggctttataC
IWGSC_CSS_3B_scaff_10592217	Cadenza0580	2994	C	T	het	—	acagcagtatcaagcccctC	acagcagtatcaagcccctT	tgatactgttgTggCggagG
IWGSC_CSS_3DS_scaff_2596771	Cadenza0580	1037	G	A	het	het	tggttatgCAcaggataatCagG	tggttatgCAcaggataatCagA	tggcaaatgtgatgtcattaggT
IWGSC_CSS_4AL_scaff_7093953	Cadenza0580	9881	C	T	hom	hom	GacaggaagccggtaacaC	GacaggaagccggtaacaT	ctccAgcaggcatgggaT
IWGSC_CSS_4BL_scaff_7037448	Cadenza0580	1837	C	T	hom	hom	CgttgaaaaGctgcaagaacttaaC	CgttgaaaaGctgcaagaacttaaT	cagttcttccTtCaGagcagataT
IWGSC_CSS_4BS_scaff_4929479	Cadenza0580	10668	G	A	hom	—	tggattttcccgcactgttC	tggattttcccgcactgttT	gtaaacaaggcatttcaagagtcA
IWGSC_CSS_4DL_scaff_14359838	Cadenza0580	1408	G	A	hom	—	gCtcAttcagggatTGTcCtaTatG	gCtcAttcagggatTGTcCtaTatA	tgaCagaacagttggtcatacT
IWGSC_CSS_4DS_scaff_2276484	Cadenza0580	8034	G	A	hom	hom	gccgtggttgatggAgaG	gccgtggttgatggAgaA	cgtccagattactgatacttgcA
IWGSC_CSS_5AL_scaff_2756579	Cadenza0580	5278	G	A	het	het	tgaatggatttttcgtcccgttC	tgaatggatttttcgtcccgttT	ggAAtCCTATgCAgaAgAaaCTG
IWGSC_CSS_5BL_scaff_10787208	Cadenza0580	10627	G	A	het	—	gcctctcacatgcggagaC	gcctctcacatgcggagaT	acgatgtcAggtggGcgT
IWGSC_CSS_5BS_scaff_2282179	Cadenza0580	5267	G	A	het	—	tgatgggctacgacgtgC	tgatgggctacgacgtgT	tcggcgcccttgaaAtcC
IWGSC_CSS_5DL_scaff_4498073	Cadenza0423	4937	C	T	hom	hom	gcaccctctggttggtcatC	gcaccctctggttggtcatT	tgagcagcaAagcagccG
IWGSC_CSS_5DS_scaff_2738970	Cadenza0423	2319	C	T	het	—	cgtgaggtgggtgatttgC	cgtgaggtgggtgatttgT	tggaactagttacactgcagtTC
IWGSC_CSS_6AL_scaff_5757109	Cadenza0423	2788	G	A	hom	hom	caggaGcctggcaaataaaGG	caggaGcctggcaaataaaGA	ctttcGcagtctcttagtttcG
IWGSC_CSS_6AS_scaff_4387871	Cadenza0423	2543	G	A	hom	hom	gcatgctaacaggcgaaaagG	gcatgctaacaggcgaaaagA	ctcatgctcctgatcttaaggtT
IWGSC_CSS_6BL_scaff_4271391	Cadenza0423	4660	C	T	hom	hom	tacgtgcatgatgtggtagtcgtaC	tacgtgcatgatgtggtagtcgtaT	gtttgaagtgcatcagatgTaccA
IWGSC_CSS_6DS_scaff_1880206	Cadenza0423	9159	G	A	het	het	ctgCgaaggctccacaaG	ctgCgaaggctccacaaA	ggatgagaagtttgcattgctC
IWGSC_CSS_7AS_scaff_4227506	Cadenza0423	952	G	A	het	—	ccatgtgtttccaatgttagagC	ccatgtgtttccaatgttagagT	tgccctagctggtatgcT
IWGSC_CSS_7BL_scaff_6681782	Cadenza0423	1486	C	T	hom	hom	agtaagCGtgacagcaatggG	agtaagCGtgacagcaatggA	AtgtctTtgGtggaagtacatcA
IWGSC_CSS_7BS_scaff_3160328	Cadenza0423	7801	C	T	het	het	tgttaaatGatacagCctgcagC	tgttaaatGatacagCctgcagT	tggaatggtgCgttgttttT
IWGSC_CSS_7DS_scaff_407428	Cadenza0423	2051	G	A	het	het	gtcGCgccatcctgacaG	gtcGCgccatcctgacaA	actcatcAggtcagcccaA
IWGSC_CSS_3AL_scaff_442479	Cadenza0364	3198	C	T	het	het	gagtcaTtaagttggtaagattggC	gagtcaTtaagttggtaagattggT	GCaGaTaaCaacaggatcacG
IWGSC_CSS_3AL_scaff_4447942	Cadenza0364	11917	G	A	het	het	gtcataaagattgctcctgtgaaG	gtcataaagattgctcctgtgaaA	ctcGgatgtgggaggaagA
IWGSC_CSS_3AS_scaff_1557483	Cadenza0364	2547	C	T	het	het	aaagtcacatcatgcttaccataaG	aaagtcacatcatgcttaccataaA	cgaaatccaacgcctcatcA
IWGSC_CSS_3AS_scaff_2648747	Cadenza0364	2688	G	A	het	het	tggAagcAcaaggggccC	tggAagcAcaaggggccT	GccgccgatggagactcG
IWGSC_CSS_3AS_scaff_3304956	Cadenza0364	1017	G	A	het	het	gtcccttgcacacagctttG	gtcccttgcacacagctttA	cctgctggactacaacttcaaT
IWGSC_CSS_3AS_scaff_3321091	Cadenza0364	4585	C	T	het	het	caagaatgATgctgatgttggaG	caagaatgATgctgatgttggaA	acatgctgaatcgccgaatC
IWGSC_CSS_3AS_scaff_3371333	Cadenza0364	538	G	A	het	het	gggaaaCgAgAcgagcgG	gggaaaCgAgAcgagcgA	ccgtgccttcctcacccT
IWGSC_CSS_3AS_scaff_3371815	Cadenza0364	1061	C	T	het	het	atccccacggcacagagG	atccccacggcacagagA	aAttggcccttggtgattcC
IWGSC_CSS_3AS_scaff_3440912	Cadenza0364	4498	G	A	het	het	ccgtaaaactttctgtgcttgC	ccgtaaaactttctgtgcttgT	atActgacaaactacatgatgtgC
IWGSC_CSS_3B_scaff_10343586	Cadenza0364	2242	G	A	het	—	ggttcTgTcctctcttccactG	ggttcTgTcctctcttccactA	tgtgttgaacccgcaagcA
IWGSC_CSS_3AL_scaff_442479	Cadenza0364	3198	C	T	het	het	gagtcaTtaagttggtaagattggC	gagtcaTtaagttggtaagattggT	GCaGaTaaCaacaggatcacG
IWGSC_CSS_3AL_scaff_4447942	Cadenza0364	11917	G	A	het	het	gtcataaagattgctcctgtgaaG	gtcataaagattgctcctgtgaaA	ctcGgatgtgggaggaagA
IWGSC_CSS_3AS_scaff_1557483	Cadenza0364	2547	C	T	het	het	aaagtcacatcatgcttaccataaG	aaagtcacatcatgcttaccataaA	cgaaatccaacgcctcatcA
IWGSC_CSS_3AS_scaff_2648747	Cadenza0364	2688	G	A	het	het	tggAagcAcaaggggccC	tggAagcAcaaggggccT	GccgccgatggagactcG
IWGSC_CSS_3AS_scaff_3304956	Cadenza0364	1017	G	A	het	het	gtcccttgcacacagctttG	gtcccttgcacacagctttA	cctgctggactacaacttcaaT
IWGSC_CSS_3AS_scaff_3321091	Cadenza0364	4585	C	T	het	het	caagaatgATgctgatgttggaG	caagaatgATgctgatgttggaA	acatgctgaatcgccgaatC

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

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

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

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

IWGSC contig	Line	Pos	WT	Mut	Predicted	M_4	Primer 1 (Kronos)	Primer 2 (mutant)	Common Primer
IWGSC_CSS_1AS_scaff_3284790	Kronos3085	7449	G	A	Het	Het	ccacaccttgagcctcgC	ccacaccttgagcctcgT	gtgattttgccaggggagA
IWGSC_CSS_1BL_scaff_3897513	Kronos3085	1515	C	T	Het	Het	gcttccactGggtcctgC	gcttccactGggtcctgT	acAaggactgcttcagaGaC
IWGSC_CSS_2AL_scaff_6434745	Kronos3085	3424	C	T	Het	Het	cctcGgttttgcaaatttctatgC	cctcGgttttgcaaatttctatgT	gGCaaTggcataacaacagatA
IWGSC_CSS_3AS_scaff_3408995	Kronos3085	732	C	T	Het	Het	aggccatttcgaattccgC	aggccatttcgaattccgT	ggTgttaTccagAacctgagTG
IWGSC_CSS_3B_scaff_10708748	Kronos3085	2675	G	A	Het	Het	gttgcatgcttcacccagG	gttgcatgcttcacccagA	gtaacaatctgagttcgtagcaC
IWGSC_CSS_4AL_scaff_7132733	Kronos3085	1799	C	T	Hom	Hom	cacccgtgagtgaccctC	cacccgtgagtgaccctT	aCcGcctaGaaagaaagcttC

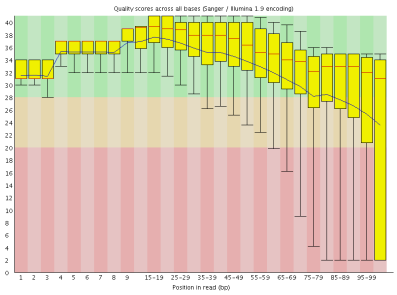
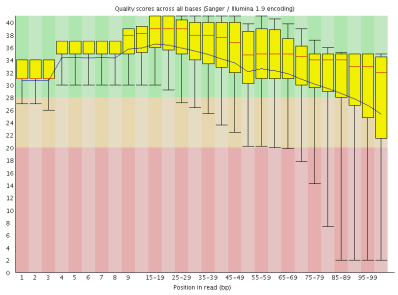
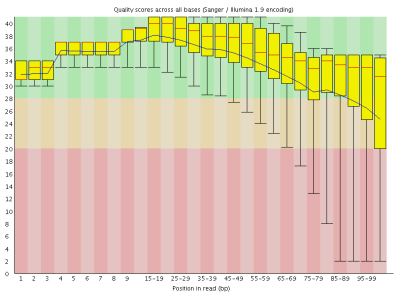
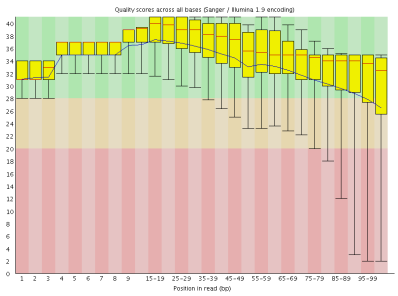
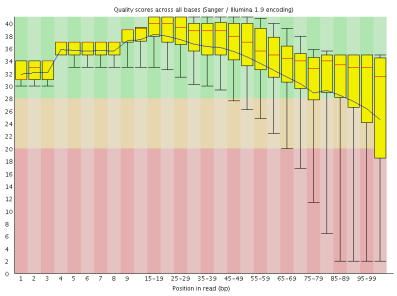
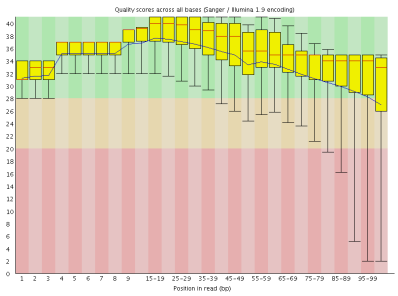
IWGSC contig	Line	Pos	WT	Mut	Predicted	M_4	Primer 1 (Kronos)	Primer 2 (mutant)	Common Primer
IWGSC_CSS_5AS_scaff_1534693	Kronos3085	4605	C	T	Het	Het	cagcttcctggccctcAtC	cagcttcctggccctcAtT	gtaCctcacgAgtcaTgagAG
IWGSC_CSS_6AS_scaff_4361911	Kronos3085	8857	G	A	Het	Het	tcacgaaagacgacttcaacctcC	tcacgaaagacgacttcaacctcT	catgaggtgctgcatctccatcA
IWGSC_CSS_6BS_scaff_3008326	Kronos3085	1528	G	A	Het	Het	ccatgttgtactggtggtgC	ccatgttgtactggtggtgT	ggaagcatggCaagtgcA
IWGSC_CSS_7AS_scaff_4214385	Kronos3085	27835	C	T	Hom	Hom	cgtaccttcgttgggaaagG	cgtaccttcgttgggaaagA	ctcttggtcagctgtataagacT
IWGSC_CSS_1AL_scaff_3929964	Kronos3191	1336	C	T	Het	Het	tttcggccatacctgacatC	tttcggccatacctgacatT	attgcctccagttcttgcaG
IWGSC_CSS_1BL_scaff_3899789	Kronos3191	7925	C	T	Het	Het	actctcacTggcagcagC	actctcacTggcagcagT	caacgtggtgcccatcGtA
IWGSC_CSS_2AL_scaff_6426728	Kronos3191	1481	G	A	Hom	Hom	gaaActgccgcagctCgC	gaaActgccgcagctCgT	ccaGcaGctcgtgagaaA
IWGSC_CSS_2BL_scaff_7960273	Kronos3191	690	C	T	Hom	Hom	gccattcatccttaggcgC	gccattcatccttaggcgT	acatgcaattgctgatgactG
IWGSC_CSS_3AS_scaff_3286603	Kronos3191	2975	G	A	Het*	Hom	ccgtgtggtttgttgtggG	ccgtgtggtttgttgtggA	gaaaggaacgtgTcaTgcaG
IWGSC_CSS_5AL_scaff_2694249	Kronos3191	2399	C	T	Het	Het	gccttccagatagagccGC	gccttccagatagagccGT	cgccacatcgacattcctG
IWGSC_CSS_5BL_scaff_10923577	Kronos3191	3713	C	T	Het	Het	gtggattgcctgagcttgC	gtggattgcctgagcttgT	tggtggccttcttgggaC
IWGSC_CSS_6AL_scaff_5823017	Kronos3191	13225	C	T	Hom	Hom	ccctttcgagcctctggaG	ccctttcgagcctctggaA	ttcgagaaggcccatcgA
IWGSC_CSS_6BS_scaff_2955394	Kronos3191	1622	C	T	Het*	Hom	gtggagatgaaggtctagcaaG	gtggagatgaaggtctagcaaA	gatactcgTgcaatgggtgT
IWGSC_CSS_7BL_scaff_6739382	Kronos3191	12261	G	A	Hom	Hom	gagacaagctttgaattgctcC	gagacaagctttgaattgctcT	CgagtgacctTcatttcccG
IWGSC_CSS_1AS_scaff_3276389	Kronos3288	9720	C	T	Hom	Hom	aCcaGcaggaccAatgtctC	aCcaGcaggaccAatgtctT	atgatgcaacctcagccaT
IWGSC_CSS_2AL_scaff_6367515	Kronos3288	6976	G	A	Het	Het	caggtcgagTgtctccgG	caggtcgagTgtctccgA	ggggtgatCtggaagggC
IWGSC_CSS_2AL_scaff_6422019	Kronos3288	4523	G	A	Het	Het	cgctaggtccctgcatagG	cgctaggtccctgcatagA	acgcAcgctaagccgtaC
IWGSC_CSS_3AL_scaff_4284850	Kronos3288	7901	C	T	Hom	Hom	tggctttggacaacatcgG	tggctttggacaacatcgA	tgtcAgcatcgacagccaG
IWGSC_CSS_4AS_scaff_5962359	Kronos3288	13049	G	A	Het	Hom	ccatcaagaagtacgagttcgaC	ccatcaagaagtacgagttcgaT	accatgcccagcttgtcA
IWGSC_CSS_6AL_scaff_5778773	Kronos3288	6853	G	A	Het	Het	gagtgaccttcccgtctttC	gagtgaccttcccgtctttT	ggagaacagctactcggcT
IWGSC_CSS_6AS_scaff_4392100	Kronos3288	3434	C	T	Het	Het	atggaagcacaggtgaccG	atggaagcacaggtgaccA	ggAagcgaaagtgaacaaacA
IWGSC_CSS_7BL_scaff_6744240	Kronos3288	9772	G	A	Het	Het	agctgttcttctcctacttcaaG	agctgttcttctcctacttcaaA	caggtcgttcttgagctcC
IWGSC_CSS_1AL_scaff_3887185	Kronos3413	9708	C	T	Hom	Hom	gcacgcctttatcgaggtaaaG	gcacgcctttatcgaggtaaaA	AgaaacagcagagcgcaA
IWGSC_CSS_2BS_scaff_3381362	Kronos3413	5160	C	T	Het*	Hom	caacttctgggctgtagtgtG	caacttctgggctgtagtgtA	tgAgaattctgacGcaaaagaC
IWGSC_CSS_3AS_scaff_3296605	Kronos3413	6154	G	A	Het	Het	ctggtcacgggctctagC	ctggtcacgggctctagT	cagcactgagagacatggaC
IWGSC_CSS_3B_scaff_10693516	Kronos3413	12632	C	T	Het	Het	ctaggcttggacaaacaggC	ctaggcttggacaaacaggT	agcttgcatctatgggcatT
IWGSC_CSS_5AS_scaff_1547699	Kronos3413	2686	G	A	Het	Het	gCtacaaccttcaccaatcgC	gCtacaaccttcaccaatcgT	gacggctttgaagtgtcatC
IWGSC_CSS_5BL_scaff_10856077	Kronos3413	5853	G	A	Het	Het	agagcttcaccccatgctC	agagcttcaccccatgctT	acgCacatttAatagctgaagC
IWGSC_CSS_6AL_scaff_5750718	Kronos3413	11046	G	A	Hom	Hom	cacgcTtcccgacttcttataG	cacgcTtcccgacttcttataA	AgacgatgtgatcaggattcaG
IWGSC_CSS_7AL_scaff_4433177	Kronos3413	3511	C	T	Het	Het	GaTgctccGtcaggctgG	GaTgctccGtcaggctgA	cactactggacaagctcttgG
IWGSC_CSS_7BL_scaff_6742567	Kronos3413	667	C	T	Het	Het	gttgcttgcgtggcagaC	gttgcttgcgtggcagaT	cattttgcaccgtgtgtcTG
IWGSC_CSS_1AL_scaff_3976389	Kronos3935	10941	C	T	Hom	Hom	ggtgaggagatcggCgatG	ggtgaggagatcggCgatA	cagtcatctacatgagaggtcaG
IWGSC_CSS_1BL_scaff_3873362	Kronos3935	1392	G	A	Het	Het	cagatctgaagcctaGcacatG	cagatctgaagcctaGcacatA	actaccagaatcagcacaaaaAC
IWGSC_CSS_2BL_scaff_7882382	Kronos3935	2721	C	T	Het	Het	gcaagctaagatgtaccgtagC	gcaagctaagatgtaccgtagT	gccacagtaggagaaagactT
IWGSC_CSS_3AL_scaff_4242376	Kronos3935	2410	C	T	Het	Het	agaacccaaaacccgTacttaG	agaacccaaaacccgTacttaA	gtagGgtCcatcCtaaagcttG
IWGSC_CSS_3B_scaff_10485067	Kronos3935	3349	C	T	Hom	Hom	gcttgagcaactactccaactG	gcttgagcaactactccaactA	gcaatttcctttaTccgcagT
IWGSC_CSS_4AS_scaff_5984153	Kronos3935	6006	G	A	Het	Het	agCaggtctggccaagttG	agCaggtctggccaagttA	cgaatGtatgaGtaggcgcT
IWGSC_CSS_4BL_scaff_7019402	Kronos3935	9081	C	T	Het	Het	tgcaatcatgtagtgagctgG	tgcaatcatgtagtgagctgA	agcatgatccctagaaCcataC
IWGSC_CSS_5BL_scaff_10842786	Kronos3935	3304	G	A	Het	Het	tggttcccGaagcctgaaC	tggttcccGaagcctgaaT	cgcatacttgaaacaTGagcAC
IWGSC_CSS_6BS_scaff_3045205	Kronos3935	2293	G	A	Het	Het	aaggaccaagcccaaactctcG	aaggaccaagcccaaactctcA	agtgatcaagcccaatgtcgcA

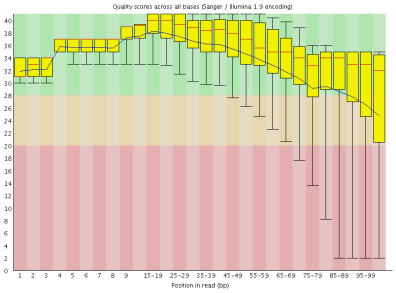
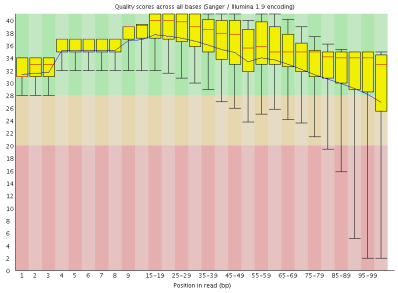
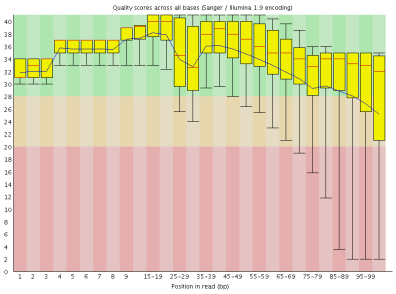
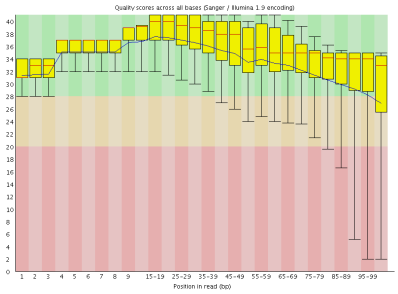
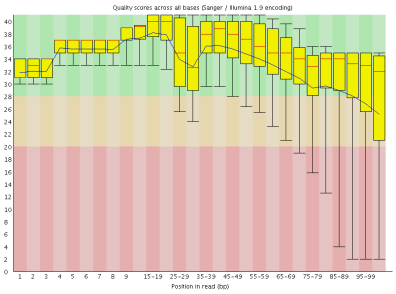
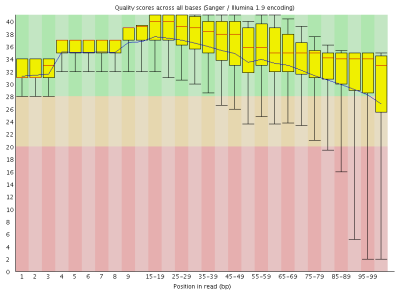
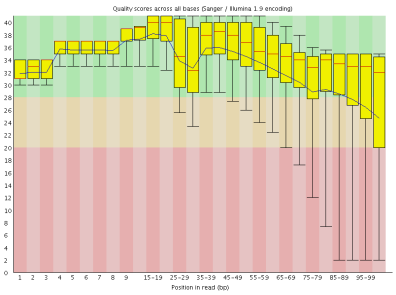
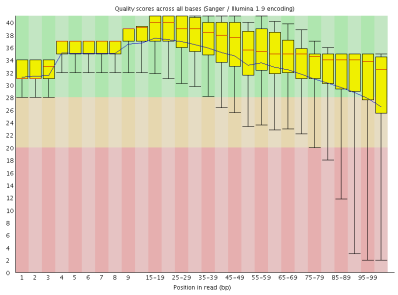
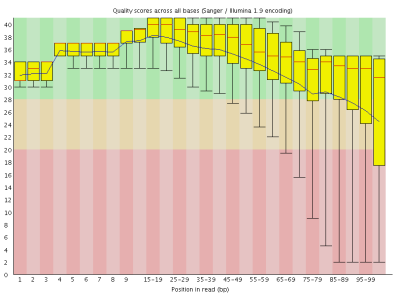
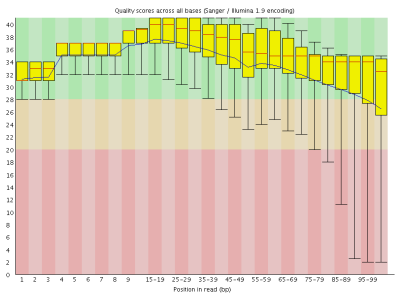
IWGSC contig	Line	Pos	WT	Mut	Predicted	M_4	Primer 1 (Kronos)	Primer 2 (mutant)	Common Primer
IWGSC_CSS_7AL_scaff_4555249	Kronos3935	4487	C	T	Het	Het	cAgtgctcgagatggcgC	cAgtgctcgagatggcgT	cCttgcaaccctcctgatT
IWGSC_CSS_1BL_scaff_3918498	Kronos4240	6096	G	A	Het	Het	ttgcatgccccaagaagaG	ttgcatgccccaagaagaA	tgggcgaactggtaatgtgG
IWGSC_CSS_2BS_scaff_5131713	Kronos4240	5900	G	A	Het	Het	cctttatcgaggaaagagacacC	cctttatcgaggaaagagacacT	caccattgtagggttccttTttC
IWGSC_CSS_5AL_scaff_2769540	Kronos4240	9626	C	T	Het	Het	tgCagtgtgggaaacggaG	tgCagtgtgggaaacggaA	catgagtGagatcttcctgcT
IWGSC_CSS_5BL_scaff_10871091	Kronos4240	7062	G	A	Het	Het	gccaaggAaccataacctgC	gccaaggAaccataacctgT	GgactcttggcAaccggA
IWGSC_CSS_6AL_scaff_5800333	Kronos4240	2360	G	A	Het	Het	cgacaggattgtgagCgC	cgacaggattgtgagCgT	tcagatgctgcaagattcatcT
IWGSC_CSS_7BL_scaff_6716931	Kronos4240	2613	G	A	Het	Het	gGtgGgtattTgcttggtgaG	gGtgGgtattTgcttggtgaA	tgGtggactcgacaGtGtA
IWGSC_CSS_2BL_scaff_8029221	Kronos4346	2860	G	A	Het	Het	tgcttccgctcttgctcC	tgcttccgctcttgctcT	atTtgcatTCgAtcgggcC
IWGSC_CSS_3B_scaff_10460714	Kronos4346	14359	C	T	Hom	Hom	ctaccttgccatgcgacatG	ctaccttgccatgcgacatA	agcaccccagtctttgacG
IWGSC_CSS_4AS_scaff_5989735	Kronos4346	6404	G	A	Hom	Hom	acgcatgctaacatcagcC	acgcatgctaacatcagcT	actcaagataccaCcgcacG
IWGSC_CSS_5BL_scaff_7648030	Kronos4346	6893	C	T	Het	Het	taccctttcctactggcagG	taccctttcctactggcagA	ttttcagaggaacacaggtatcA
IWGSC_CSS_6AL_scaff_5755840	Kronos4346	778	C	T	Het	Het	atcgagtaagctgtcacCgC	atcgagtaagctgtcacCgT	acctgcatgtcaCatccaC
IWGSC_CSS_6BS_scaff_2972151	Kronos4346	7876	G	A	Hom	Hom	gcagcaatgtcActgtttgG	gcagcaatgtcActgtttgA	gcttggactgggcatttatG
IWGSC_CSS_7AL_scaff_4542983	Kronos4346	18700	G	A	Het	Het	gcagggctAccggatacC	gcagggctAccggatacT	catctgccGgttaaacatgC
IWGSC_CSS_7BS_scaff_3098098	Kronos4346	5183	C	T	Het	Het	gCgatatggtacttgcaatgaG	gCgatatggtacttgcaatgaA	ttacattgcttataGTttgCcgG
IWGSC_CSS_1AS_scaff_3259804	Kronos4485	219	C	T	Het	Het	gtcggcacaacccctgC	gtcggcacaacccctgT	gcttctttaaggagggcgA
IWGSC_CSS_2AL_scaff_6315418	Kronos4485	10490	G	A	Hom	Hom	gcccctctcaaCcttctcagC	gcccctctcaaCcttctcagT	ttcagacgctCgaggaatttccC
IWGSC_CSS_2BS_scaff_5181092	Kronos4485	3742	G	A	Het	Het	TggccagcacacctgcaG	TggccagcacacctgcaA	tggacgatgagTgatggAaaT
IWGSC_CSS_3B_scaff_10425015	Kronos4485	2372	C	T	Het	Het	gctactgaagttggCtcGG	gctactgaagttggCtcGA	cttcacatccttgggggTtC
IWGSC_CSS_3B_scaff_10775915	Kronos4485	4701	C	T	Het	Het	ccaagggctgcagagagG	ccaagggctgcagagagA	agacctcacgatGtcctcC
IWGSC_CSS_5AL_scaff_2754304	Kronos4485	2301	G	A	Het	Het	taacccTgccatcgcccG	taacccTgccatcgcccA	cattgGccagccaTgacT
IWGSC_CSS_5BL_scaff_10919959	Kronos4485	1867	C	T	Hom	Hom	gatgccctttgtggagaagG	gatgccctttgtggagaagA	tcttgttcccgaaacatgtcA
IWGSC_CSS_7AS_scaff_4245431	Kronos4485	3402	G	A	Hom	Hom	aaggcgcctggtgtttcC	aaggcgcctggtgtttcT	agtaagtggaAcagctaagatcaT
IWGSC_CSS_7BL_scaff_6667357	Kronos4485	641	C	T	Het	Het	gatcAgctgctcattcgagG	gatcAgctgctcattcgagA	ttccctgtcaattgatgccC

Appendix B

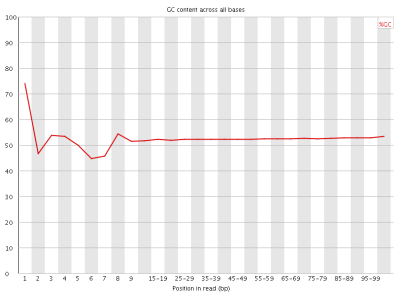
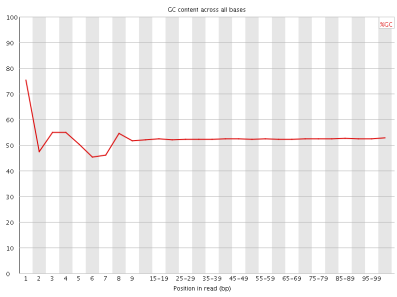
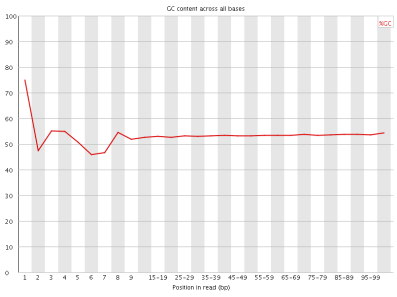
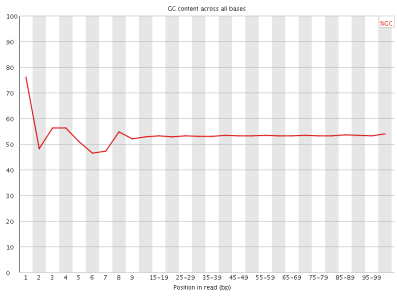
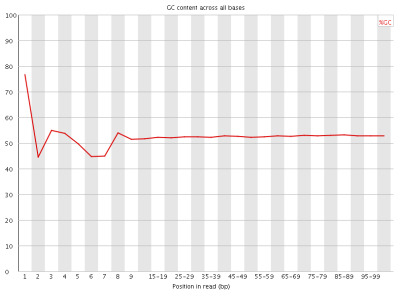
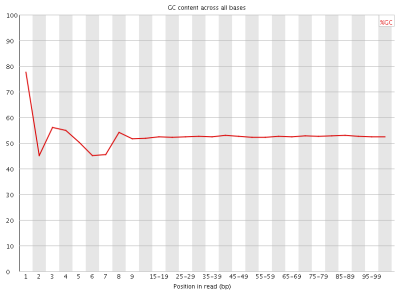
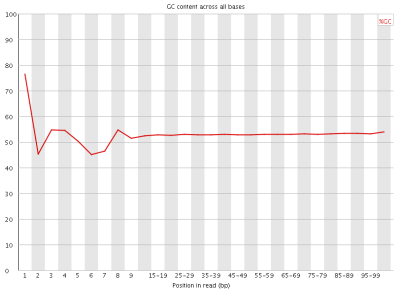
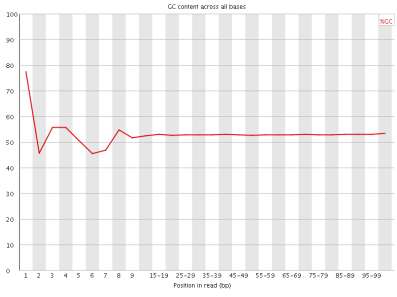
Quality Control

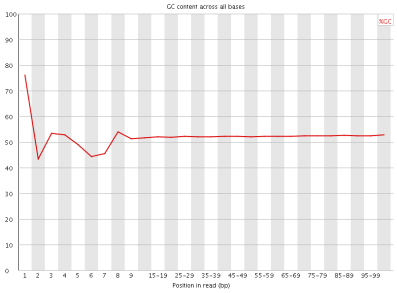
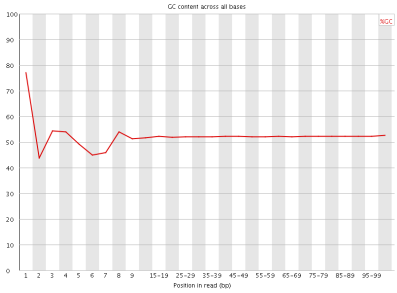
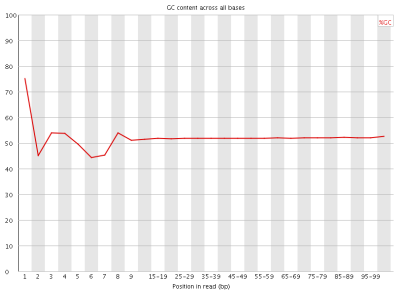
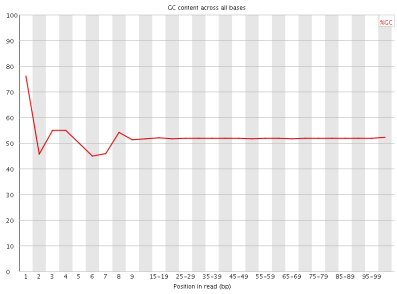
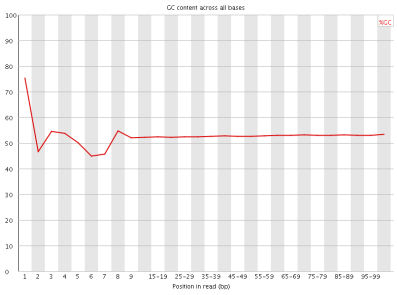
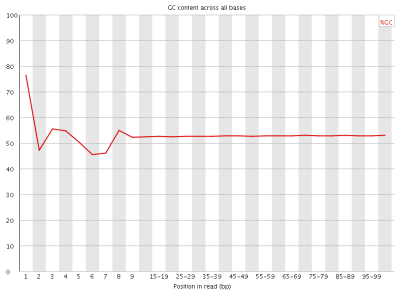
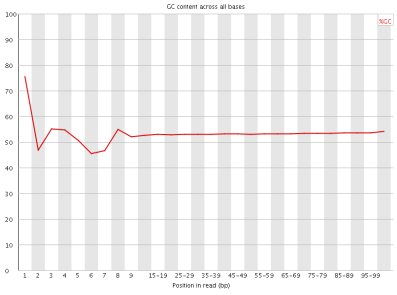
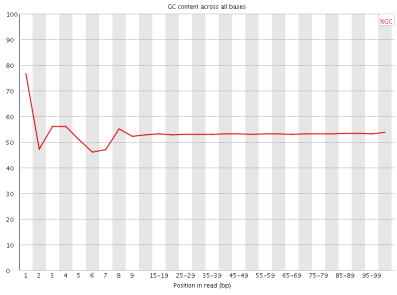
B.1 Sequence read quality

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

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

B.2 Sequence GC content

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

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

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