UNIVERSIDADE FEDERAL DE PELOTAS

Faculdade de Agronomia Eliseu Maciel

Programa de Pós-Graduação em Agronomia

Tese

Introgressões de *Triticum timopheevii* em germoplasma brasileiro de trigo

Mariana Peil da Rosa

Pelotas, 2018

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Tese apresentada ao Programa de Pós-Graduação em Agronomia da Faculdade de Agronomia Eliseu Maciel da Universidade Federal de Pelotas, como requisito parcial à obtenção do título de Doutor em Agronomia.

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Pelotas, 2018

Universidade Federal de Pelotas / Sistema de Bibliotecas Catalogação na Publicação

R788i Rosa, Mariana Peil da

Introgressões de *Triticum timopheevii* em germoplasma brasileiro de trigo / Mariana Peil da Rosa ; Antonio Costa de Oliveira, orientador ; Surbhi Grewal, coorientador. — Pelotas, 2018.

94 f. : il.

Tese (Doutorado) — Programa de Pós-Graduação em Agronomia, Faculdade de Agronomia Eliseu Maciel, Universidade Federal de Pelotas, 2018.

1. Variabilidade genética. 2. Biotecnologia. 3. Resistência a doenças. I. Oliveira, Antonio Costa de, orient.
II. Grewal, Surbhi, coorient. III. Título.

CDD : 633.11

Elaborada por Dafne Silva de Freitas CRB: 10/2175

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Tese aprovada, como requisito parcial, para obtenção do grau de Doutor em Ciências, Programa de Pós-Graduação em Agronomia, Faculdade de Agronomia Eliseu Maciel, Universidade Federal de Pelotas.

Data da Defesa: 20 de agosto de 2018

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À minha mãe Maria Cristina

Dedico

Agradecimentos

À minha mãe que sempre incentivou os meus estudos, e me ensinou que a educação é a maior herança que se pode deixar, e incansavelmente me deu todo o suporte, e amor incondicional para conseguir realizar meus sonhos.

Ao meu querido irmão Carlos Henrique por todo amor, apoio e compreensão e ao longo desses anos de estudo.

Aos meus avós por sempre estarem ao meu lado e se orgulharem da minha trajetória e às minhas tias Marias; Elisa, Ignez e Thereza que também foram minhas mães, perto ou longe estiveram presentes em todos os passos de minha vida e comemoram a cada conquista que eu alcancei.

Ao meu namorado Guilherme pelo companheirismo, amizade, amor e carinho, por apoiar e me fazer feliz mesmo nos momentos difíceis.

Ao meu orientador Antonio Oliveira pela orientação e compreensão, por todas as oportunidades oferecidas durante esses anos e por me proporcionar participar desse projeto o qual tenho muito apreço.

Aos colegas que se tornaram amigos durante essa trajetória; Fabiane Igansi, Danyela de Cássia, Carolina Leiva, Renata Ahlert, Lílian Barros, Cézar Verdi, Vianei Rotter, com certeza tudo torna mais fácil quando temos os amigos do lado e não imagino como seria meu doutorado sem a presença de vocês.

Aos professores Ian e Julie King e aos técnicos Caiyun Yang e Duncan Scholefield, da Universidade de Nottingham que disponibilizaram seu tempo com muita dedicação para o desenvolvimento do meu projeto.

À minha coorientadora Surbhi Grewal pela impecável orientação e amizade que levarei para a vida inteira. À Manel Othmeni por ser a minha família quando estávamos longe de casa.

Aos colegas Glacy Jaqueline da Silva e Eduardo Venske por executarem esse projeto junto comigo.

Aos demais colegas e professores do CGF por contribuírem com a minha formação.

À CAPES, CNPQ pelas bolsas concedidas e ao BBSRC por financiar este projeto.

“You can't build a peaceful world on empty stomachs and human misery”.

Norman Borlaug

Resumo

DA ROSA, Mariana Peil. **Introgressões de *Triticum timopheevii* em germoplasma brasileiro de trigo.** 2018. 94p. Tese (Doutorado) – Programa de Pós-graduação em Agronomia. Universidade Federal de Pelotas, Pelotas, 2018.

O aumento da produção no Brasil, assim como no mundo, é uma estratégia para segurança alimentar. No entanto, uma estagnação nos aumentos de produtividade devido, entre outros fatores, a redução da variabilidade genética através da seleção ao passar dos anos. *Triticum timopheevii* Zhuk. (2n = 28, composição genômica A^tA^tGG) é um trigo tetraploide e possui alelos desejáveis, principalmente para resistência a doenças, e ele pode ser usado para ampliar variabilidade genética no trigo. Este estudo propôs realizar a introgressão de segmentos de DNA de *T. timopheevii*. em uma cultivar elite de trigo brasileira. Linhas de *T. aestivum*/*T. timopheevii* na geração RC_2 foram polinizadas com TBIO Sinuelo (TBIO) e um arranjo de genotipagem *Affymetrix Axiom Array* juntamente com hibridização genômica *in situ* (GISH) foram utilizados para detectar e caracterizar as introgressões. No total, 548 cruzamentos foram realizados, e 4.579 sementes foram produzidas possibilitando a geração de um mapa de ligação do *T. timopheevii*. Este mapa foi usado para identificar 316 introgressões putativas nos híbridos trigo/*T. Timopheevii.* Foram encontradas introgressões dos grupos de ligação. A análise comparativa confirmou as translocações $4A^tL/5A^tL$ herdadas do *T. urartu* e já reportadas no *T. timopheevii* e as translocações específicas da espécie $6A^tS/1GS$ $3A^tL/4A^tL$ presentes no *T. timopheevii.* Os dados obtidos aqui mostram que *T. timopheevii* tem um grande potencial para ser usado em programas de melhoramento porque suas linhas de introgressão apresentam altas taxas de germinação alta fertilidade e boa produção de sementes.

Palavras-chave: variabilidade genética, biotecnologia, resistência a doenças, SNPs

Abstract

DA ROSA, Mariana Peil. ***Triticum timopheevii* introgressions into a Brazilian wheat germplasm.** 2018. 94p. Thesis (Doctoral degree) – Programa de Pós-graduação em Agronomia. Universidade Federal de Pelotas, Pelotas, 2018.

Increasing wheat yield in Brazil, as well as in the world, is a strategy for food security. However, a stagnation has been observed, due to, among other things, the reduction of genetic variability through selection over the decades. *Triticum timopheevii* Zhuk. (2n = 28, genome composition A^tA^tGG) is a tetraploid wheat which has desirable alleles, mainly for disease resistance, and it can be used to increase genetic variability in wheat. This study proposed performing the introgression of DNA segments from *T. timopheevii* lines into a Brazilian elite cultivar. Wheat/*T. timopheevii* lines in BC_2 generation were pollinated with TBIO Sinuelo (TBIO) and Affymetrix Axiom Array along with genomic *in situ* hybridisation (GISH) were used to detect and characterize introgressions. In total, 548 crosses were performed, and 4,579 seeds were produced enabling the generation of a linkage map of *T. timopheevii*. This was used to identify 316 *T. aestivum*/*T. timopheevii* putative introgressions. Introgressions of all 14 groups of *T. timopheevii* were found. Comparative analysis showed that *T. timopheevii* has the $4A^tL/5A^tL$ translocations inherited from *T. urartu* and the species-specific $6A^tS/1GS$, $3A^tL/4A^tL$ already reported in *T. timopheevii.* From the data obtained *T. timopheevii* has good potential to be used in introgression programs because its introgression lines present high germination rates, fertility level and good seed production.

Key-words: genetic diversity, biotechnology, disease resistance, SNPs

List of figures

List of tables

List of abreviations

ADAP	Axiom® Data Analysis pipeline
AFLP	Amplified Fragment Length Polymorphisms
APT	Affymetrix powertools
BBSRC	Biotechnology and Biological Sciences Research Council
BCx	Backcross
CAPES	Coordenação de Aperfeiçoamento de Pessoal de Nível Superior
CGF	Centro de Genômica e Fitomelhoramento
cM	centiMorgan
CNPQ	Conselho Nacional de Desenvolvimento Científico e Tecnológico
COS	Conserved Orthologous Sequences
CRBT	Call Rate Below Threshold
CS	Chinese spring
cv.	CULTIVAR
DArT	Diversity Array Technology
DNA	DNA Deoxyribonucleic Acid
EST	Expressed Sequence Tags
FHB	Fusarium head blight
FISH	Fluorescence *in situ* hybridisation
Gb	Gigabase
GBS	Genotyping-by-Sequencing
Gc	Gametocidal genes
GGT	Graphical Genotypes
GISH	Genomic *in situ* hybridisation
ISH	*in situ* hybridisation
IWGSC	The International Wheat Genome Sequence Consortium
KASP	Kompetitive allele-specific PCR
Kb	Kilobases
LG	Linkage group
LOD	LOD Log-odds
Lr	Leaf rust
MAPA	Ministério Da Agricultura Pecuária E Abastecimento

MAS	Marker Assisted Selection
Mb	megabase
mcGISH	Multicolour GISH
MIAG	Powdery mildew
MYA	Million years ago
PCR	Polymerase chain reaction
Ph1	Pairing homoeologous 1
PHR	Polymorphic High Resolution
Pm	Powdery mildew
QTL	Quantitative Trait Locus
RAPD	Randomly Amplified Polymorphic DNAs
RFLP	Restriction Fragment Length Polymorphisms
SnbTM	Winter wheat leaf blotch
SNP	Single Nucleotide Polymorphism
Sr	Stem rust
SSR	Simple Sequence Repeats
STS	Sequence Tagged Sites
UFPel	Universidade Federal de Pelotas
USDA	United States Department of Agriculture
Yr	Yellow rust

Summary

1. Introduction

Global population is growing and it is expected to reach at least 9 billion people (FAO, 2017; FISCHER; BYERLEE; EDMEADES, 2014). It is suggested that an increase of at least 60% of food demand will be needed by 2050 to feed the world's population (OECD/FAO, 2012; TILMAN et al., 2011) . Wheat is the major staple crop worldwide (REYNOLDS et al., 2012) as it is the most widely grown and is also the main source of nutrients for the world population (CURTIS; RAJARAM; MACPHERSON, 2002; DUBCOVSKY; DVORAK, 2014). Increasing wheat yield in Brazil, as well as in the world, is a strategy for food security. However, a plateau in wheat productivity has been observed (BRISSON et al., 2010; RAY et al., 2013).

Common wheat (*Triticum aestivum*, 2n = 6x = 42, genome AABBDD) is an allohexaploid species originated from two natural interspecific hybridisation events, involving three diploid ancestors (KIHARA, 1919, 1954; KIHARA; YAMASHITA; TANAKA, 1959; SAKAMURA, 1918; SAX, 1922). Thus, since its origin, wheat is a narrow species as it descended from a very small number of interspecific hybridisations (COX, 1996), in addition, domestication, extensive plant breeding and intensive selection probably have reduced diversity among cultivars, narrowing variability for the future breeding advances (TANKSLEY; MCCOUCH, 1997).

Wild relatives have been used to introduce allelic variation into wheat germplasm as an alternative to increase variability. Wide hybridisation enables the introduction of genes for many agronomical important traits, and they can be really useful for plant breeders to develop superior cultivars with high yield and adapted to different environments (ABLE; LANGRIDGE; MILLIGAN, 2007; WANG, 2009).

Triticum timopheevii Zhuk. is an allotetraploid wheat (genome formula A^tA^tGG) and it belongs to the secondary gene pool of wheat (CHAUDHARY; KAILA; RATHER, 2013), its A^t is similar to the A from *T. turgidum* (AABB) and *T. aestivum* (AABBDD), (ABROUK et al., 2017; ALLABY; BROWN, 2000; DVORAK et al., 1993; KILIAN et al., 2007; TAKUMI et al., 1993). Genes from timopheevii can be transferred to wheat either by direct hybridisation or through the development of amphiploids (MIKÓ et al., 2014). .

Wheats from Timopheevii group carry resistance for fungal diseases (LEONOVA et al., 2011) and they have been used over the years in crosses with other species in order to transfer immunity genes. Up to date, twelve genes controlling resistance to disease were transferred from *T. timopheevii* genome into common wheat (MCINTOSH et al., 2013).

South of Brazil is the main producer region of wheat but the environment is quite unstable, the occurrence of rain and high temperatures during heading time contributes to the spread of diseases, posing the major challenges that affect the growing seasons. Fungi are main pathogens responsible for decreasing yield gain and grain quality in wheat (BORÉM; SCHEEREN, 2017).

Some examples of the diseases affecting wheat production are powdery mildew (*Blumeria graminis* f. sp. *Tritici*), leaf rust (*Puccinia triticina* Eriks.), fusarium head blight (*Fusarium graminearum*), septoria (*Mycosphaerella graminicola*), wheat head blast (*Magnaporthe oryzae Triticum* pathotype), helminthosporium blight (*Bipolaris sorokiniana*) and the reduction of yield gain can reach 62, 63, 38, 38, 50 and 80% respectively (CASA; REIS, 2014). The preferential method for controlling diseases is the usage of resistant varieties, unfortunately this resistance may not last and new races of pathogens can appear (BARCELLOS; MORAES-FERNANDES; ROELFS, 1997).

Many genes conferring resistance from the timopheevii group were already identified and transferred to common wheat, however this group may carry other unknown genes which can be used to transfer not only resistance to disease but other desirable traits. Also, the introduction of alleles from this species into a Brazilian wheat genotype was not accomplished yet and this study could bring many benefits for wheat breeding in Brazil.

The main aims of this research are to transfer chromosome segments from *T. timopheevii* into a Brazilian hexaploid wheat cv. TBIO Sinuelo, to detect and characterize the introgressions via a wheat/wild relative SNP array; to validate the genotyping with the use of genomic *in situ* hybridisation and to analyse the synteny between *T. timopheevii* and hexaploid wheat.

2. Literature review

2.1 Wheat in the world

World's population is growing, and it is estimated that by 2050 it could reach more than 9 billion people (FAO, 2017). To feed the world, global agriculture production may need to be increased by 60% - 110% (OECD/FAO, 2012; TILMAN et al., 2011), and around 2.4 % per year rate of yield gains will be needed to reach it (RAY et al., 2013). Maize, rice, wheat, and soybean are the most representative crops, together their production represents two-third of current harvested global crop calories (CASSMAN, 1999; TILMAN et al., 2011). Unfortunately, the global average rates of yield increase for these crops are 1.6%, 1.0%, 0.9% and 1.3%, respectively (RAY et al., 2013), and at the current rates, it seems the yield gain will not be achieved.

Wheat was one of the first domesticated crops and it has been a staple food for the major civilizations over the years. It is cultivated from the equator to temperate lands and it can be grown at high latitudes, the land area occupied with wheat is more than any other crop, around 220 million hectares representing 17% of all world's cultivated land (CURTIS; RAJARAM; MACPHERSON, 2002; FAO, 2016). About 20% of the calories consumed by humans is provided from wheat, and 95% it is from bread wheat (DUBCOVSKY; DVORAK, 2014; FAO, 2006), which represents the main source of income for millions of small-scale farmers living in developed countries. Increasing wheat yield gain will have more influence on global food security than that of any other crop (REYNOLDS et al., 2012).

In Brazil, hexaploid wheat occupies the third position among the most produced cereals, the annual production is around 5 million tonnes, with an average yield of 2.5 tonnes per hectare (CONAB, 2018). The national wheat consumption is around 12 million tonnes, and almost half of the wheat is imported (ABITRIGO, 2018). The Southern Region is the main producer, although there was an attempt of increasing wheat production area in the Cerrado region. Unfortunately, the need for irrigation increases the production costs, making farmers switch to more profitable crops. Increasing wheat yield should be the best alternative to increase national production.

Production can be increased by improving the yield gain per area, something that can be achieved with plant breeding and with reduction of pre and post-harvest losses. Based on genetic potential, it has been suggested the absolute yield of wheat would be 20 t ha^{-1} (HANSON; BORLAUG; ANDERSON, 1982). The current highest commercial attainable yield record is 16.79 t ha^{-1} in New Zealand (GUINNESS, 2017). Unfortunately, the wheat yield average for the world during the last ten years was just 3.3 t ha^{-1} (FAO, 2016). Closing the yield gap must be one of the major goals of organizations involved with world food policy and wheat research for the future.

Current research to improve wheat yields covers a broad strategies including the mixture of germplasms through crossing, interspecific and intergeneric crosses, biotechnology techniques, hybrid wheat, studies on the physiology of the wheat plant and on the host-plant relationships of various pests that attack it and numerous other important research avenues (CURTIS; RAJARAM; MACPHERSON, 2002).

The main aims of this research are to transfer chromosome segments from *T. timopheevii* into a Brazilian hexaploid wheat cv. TBIO Sinuelo, to detect and characterize the introgressions via a wheat/wild relative SNP array; to validate the genotyping with the use of genomic *in situ* hybridisation and to analyse the synteny between *T. timopheevii* and hexaploid wheat.

2.2 Wheat origin and evolution

The genus *Triticum* consists of six domesticated species: *Triticum monococcum* L. (A^mA^m genome); *Triticum urartu* Tumanian ex Gandilyan (AA genome); *Triticum turgidum* L. (AABB genome); *Triticum timopheevii* (Zhuk.) Zhuk. (A^tA^tGG genome); *Triticum aestivum* L. (AABBDD genome); and *Triticum zhukovskyi* Menabde & Ericz. ($A^mA^mA^tA^tGG$ genome), and they are present at the diploid, tetraploid and hexaploid levels (figure 1). The diploid AA genome species, *T. monococcum,* and *T. urartu* diverged less than one million years ago (HUANG et al., 2002).

After that, less than 0.5 million years ago, the tetraploid species *T. turgidum* (AABB genome) and *T. timopheevii* (A^tA^tGG genome) evolved through two independent hybridisation events between (diphyletic origin): the A genome from *T. urartu* (ABROUK et al., 2017; ALLABY; BROWN, 2000; DVORAK et al., 1993; KILIAN et al., 2007a; TAKUMI et al., 1993) the B and G genomes from a species closely related to *Aegilops speltoides* Tausch (SS genome) (DVORAK, 2001; MATSUOKA, 2011; SEARS, 1969). A monophyletic hypothesis exists suggesting that both species arose from a single hybridisation event (GILL; CHEN, 1987; WAGENAAR, 1961), however a number of studies show strong evidence supporting the diphyletic origin.

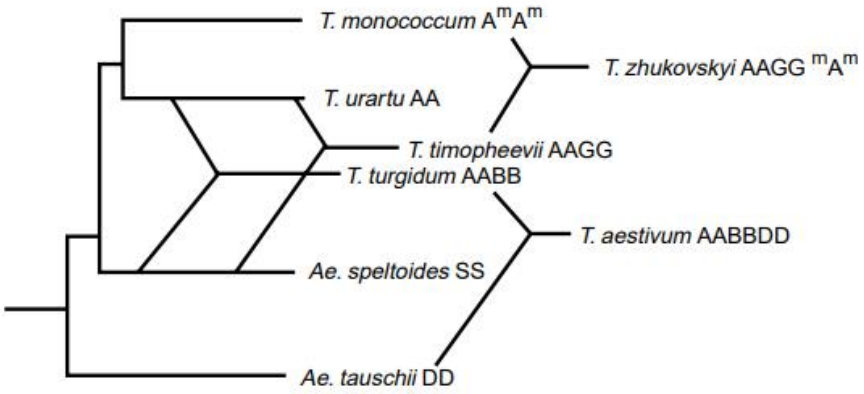

Figure 1 - Evolution of wheat.
Source: DVORAK, J., 2001.

During the development of agriculture around 10,000 years ago, *T. turgidum* and *T. timopheevii* were domesticated and their cultivated forms *T. turgidum* subsp. durum and *T. timopheevii subsp. timopheevii,* respectively, appeared (FELDMAN, 2001; SALAMINI et al., 2002). The diploid *T. monococcum* also was domesticated in the same area (KILIAN et al., 2007b; TANNO; WILCOX, 2006).

After this period, the hexaploid *Triticum* wheats emerged through natural hybridisation between the tetraploid cultivars and diploid *Aegilops* and *Triticum* species. *T. zhukovskyi* ($A^mA^mA^tA^tGG$ genome) is thought to be originated through

hybridisation of *T. timopheevii* with cultivated einkorn *T. monococcum*. *T. aestivum* (AABBDD genome) is thought to have arisen through hybridisation of *T. turgidum* with the wild wheat species *Aegilops tauschii* Coss. (DD genome) (KIHARA, 1944; MCFADDEN; SEARS, 1944). Common wheat is an allohexaploid species originated from two natural interspecific hybridisation events, involving three ancestors, followed by chromosome doubling enabling, the production of fertile plants (KIHARA, 1919, 1954; KIHARA; YAMASHITA; TANAKA, 1959; SAKAMURA, 1918; SAX, 1922).

2.3 Genetic variability in wheat

Allopolyploids arise from processes of interspecific hybridisation and chromosome doubling, then containing the entire genome of two or more species in homozygous condition. Common wheat is a species with narrow genetic bases since its origin because its germplasm has descended from a very small number of interspecific hybridisations resulted from two amphiploidisation events involving tree ancestors (COX, 1998; KIHARA, 1919, 1954; KIHARA; YAMASHITA; TANAKA, 1959; SAKAMURA, 1918; SAX, 1922).

The frequent use of few genotypes as genetic resources in wheat breeding programs and the monotonous usage of a limited number of varieties have caused serious genetic erosion in cultivated wheat (REIF et al., 2005). The polyploidisation, subsequent domestication, and inbreeding have reduced the genetic diversity in cultivated wheat compared with its wild ancestors (HAUDRY et al., 2007; TANKSLEY; MCCOUCH, 1997).

The lack of genetic diversity is a major issue for wheat breeders and it limits their ability to produce new varieties (ROUSSEL et al., 2004; WHITE et al., 2008) Cultivation of germplasm with a narrow genetic base has risks, such as mutations in pest populations or changes in environmental conditions that may bring about stresses that the cultivar could not cope with and, therefore, could lead to severe losses (REIF et al., 2005).

2.4 Homology, recombination and genome structure

Meiotic recombination is central to evolution, speciation, breeding and crop improvement. It results in the formation of chiasma at the sites of genetic crossovers, at least one chiasma between homologous chromosomes is essential for genetic recombination and accurate chromosome segregation at the first meiotic division (JONES, 1984; JONES; FRANKLIN, 2006). Polyploid organisms with multiple sets of chromosomes have additional mechanisms for distinguishing between homologous and homoeologous pairing and recombination (BREIMAN; GRAUR, 1995).

Hexaploid wheat is composed of three related ancestral genomes (A, B and D), each containing seven pairs of homologous chromosomes. (KIHARA, 1924; SAKAMURA, 1918). For hexaploid wheat to be highly fertile, it needs to have a diploid-like behavior, thus only true homologs may pair with each other, for example, 1A must pair with 1A and not with its homeologous 1B or 1D (KOO et al., 2017). The major locus controlling this pairing behavior in wheat is *Ph1* (*pairing homeologous*), which is a single dominant locus located on the long arm of the chromosome 5B (RILEY; CHAPMAN, 1958).

Mutants carrying a deletion of the *Ph1* locus exhibit a degree of pairing of related (homoeologous) chromosomes and hence multivalent formation at metaphase I (ROBERTS et al., 1999; SEARS, 1977). New deletion analysis defined the *Ph1* effect to a 2.5 Mb interstitial region inside the locus of 5BL wheat chromosome (AL-KAFF et al., 2008; GRIFFITHS et al., 2006; MARTÍN et al., 2017), and its absence delays homologous pairing allowing homoeologous instead (MARTÍN et al., 2017; REY et al., 2017).

The lack of *Ph1* effect in diploid relatives of wheat suggests that *Ph1* locus arose after polyploidisation (CHAPMAN; RILEY 1970). Restriction of pairing between homeologous ensures regular segregation, high fertility and genetic stability. Also, amphypolyploids have a buffer effect, they can tolerate structural changes e.g., translocation, that diploid cannot tolerate. Diploid mechanism of polyploids provided an abundance of redundant genes, that could diverge to achieve new functions, however, many genes become silenced and the number of functional genes was greatly reduced, so the polyploid does not necessary has more expressed genes than a diploid (BREIMAN; GRAUR, 1995).

Hexaploid wheat genome size is around 17 Gb, but each subgenome has around 5,5 Gb, with more than 80% of highly repetitive transposable elements (EILAM et al., 2007; IWGSC, 2014; WICKER et al., 2011).

2.5 Use of wild relatives to increase genetic diversity

Increasing genetic diversity in wheat depends on the genetic distance of the wild relative species from wheat genome donors. Interspecific hybridisation, also called wide hybridisation, enables the introduction of genes for many agronomically important traits, and it has been widely used by plant breeders to develop superior cultivars with high yield and adapted to different environments (ABLE; LANGRIDGE; MILLIGAN, 2007; WANG, 2009). For several decades, these wide crosses have been a reservoir of novel variation for wheat improvement.

Genetic diversity is paramount for cultivated crops. The diversity of wheat resides in three gene pools of the Triticeae structured based on the genomic constitution of the related species (Figure 2). The Primary gene pool includes the hexaploid landraces, cultivated tetraploids, wild *T. dicoccoides* and diploid donors of the A and D genomes of hexaploid wheat. The secondary gene pool consists of the polyploid *Triticum* and *Aegilops* species which share at least one genome with wheat. Wild relatives with genomes that are non-homologous to wheat reside in the tertiary gene pool that includes diploid and polyploid species of Triticeae-carrying genomes other than A, B and D. (CHAUDHARY; KAILA; RATHER, 2014; HARLAN; WET, 1971).

Gene transfer from these species to wheat will depend on the level of homology they share since the chromosome pairing and recombination is controlled by *Ph1* locus (RILEY; CHAPMAN, 1958). For species comprising the primary gene pool, standard breeding methods like hybridisation, backcrossing and selection are efficient (GILL; RAUPP, 1987; MCFADDEN; SEARS, 1946). For species from the second, gene transfer requires cytogenetic manipulations to enhance the recombination between alien and wheat homoeologous chromosomes. And for the third, physical and genetic methods that cause random chromosome breaks and promote recombination have been used in engineering transfers from the tertiary gene pool into the genetic background of cultivated wheat species (CHAUDHARY; KAILA; RATHER, 2014).

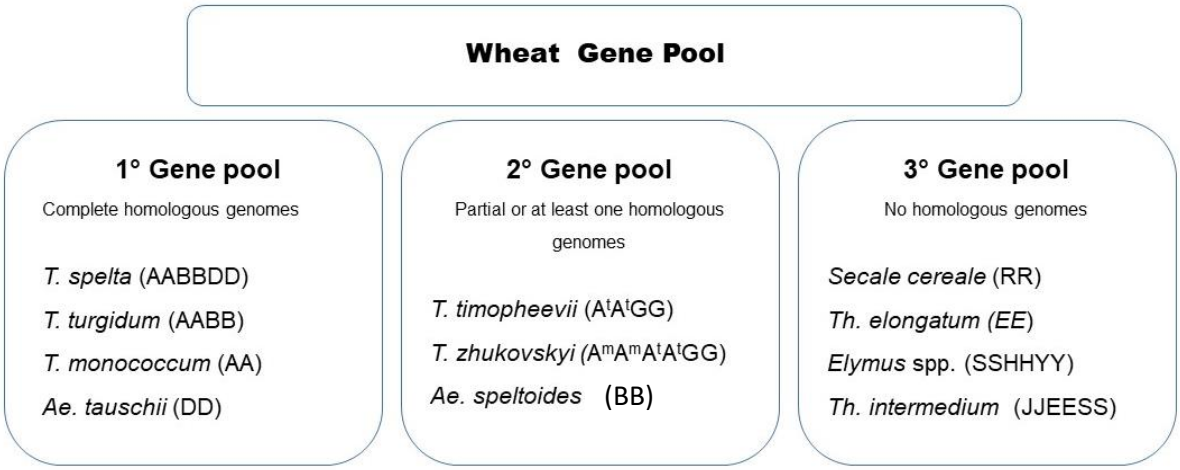

Figure 2 - Wheat gene pools and some examples.
Source: Adapted from Chaudhary et al. (2013).

Some wide species, such as *Amblyopyrum muticum* [(Boiss.) Eig. (*Aegilops mutica* Boiss.) (2n = 2x = 14; genome TT)] carry genes which suppress the *Ph1* locus, and in this case, even if they are not from the primary gene pool, recombination between homeologous can occur, thus direct crosses are efficient to transfer genes to wheat (DOVER; RILEY, 1972; KING et al., 2017). In an introgression program, understanding the species are you going to work with, is of great importance to decide the best approach to increase variability.

2.6 The use of *Triticum timopheevii* as a source of variation

Triticum timopheevii Zhuk. is an allotetraploid wheat (genome formula A^tA^tGG), it belongs to the Timopheevii group. Its A^t genome has some similarity with the A from *T. turgidum* (AABB) and *T. aestivum* (AABBDD), according to strong evidence that they derived from the same ancestral *T. monoccoccum* L. ssp. *urartu* (ALLABY; BROWN, 2000; DVORAK et al., 1993; KILIAN et al., 2007b; TAKUMI et al., 1993). Although it does not have their B genome, (SEARS, 1969), it is suggested that its G genome is also originated from *Ae. speltoides* Tausch. or an ancestral close to it (DVORAK; ZHANG, 1990; MIYASHITA; MORI; TSUNEWAKI, 1994; OGIHARA; TSUNEWAKI, 1988; SASANUMA; MIYASHITA; TSUNEWAKI, 1996).

Wheats form Timopheevii group are part of the secondary gene pool of wheat (CHAUDHARY; KAILA; RATHER, 2014), and they can be transferred into by direct hybridisation or with the use of *Ph1* mutants to enhance recombination (MIKÓ et al., 2014). Wheats from Timopheevii group carry resistance for fungal diseases (LEONOVA et al., 2011) and they have been used over the years in crosses with other species in order to transfer immunity genes.

Up to date, genes controlling resistance to: stem rust, ***Sr36*** (2BS), ***Sr37*** (4BL) (GYARFAS, 1978; MCINTOSH; GYARFAS, 1971), and ***Sr40*** (2BS) (DYCK, 1992; WU; PUMPHREY; BAI, 2009) to leaf rust, ***Lr18*** (5BL) (DYCK; SAMBORSKI, 1968; FRIEBE et al., 1996; MCINTOSH, 1983; YAMAMORI, 1994), ***Lr50*** (2BL) (BROWN-GUEDIRA; SINGH; FRITZ, 2003; LEONOVA et al., 2010), ***LrTt1*** (2A) (LEONOVA et al., 2004), and ***LrTt2*** (5BL); to powdery mildew, ***Pm6*** (2B) (HELMSJØRGENSEN; JENSEN, 1973), ***Pm27*** (6B) (JÄRVE et al., 2000), ***Pm37*** (7AL) (PERUGINI et al., 2008), and ***MIAG12*** (7AL) (MAXWELL; LYERLY; MURPHY, 2009); and to winter wheat leaf blotch, ***SnbTM*** (3AL) (MA; HUGHES, 1995) were transferred from *T. timopheevii* genome into common wheat (MCINTOSH et al., 2013).

2.7 Strategies for gene transfer

There are some strategies to introduce desired traits from wild relatives into wheat. Whole genome approach is used when the whole genome of the relative species is desired, it is accomplished by crossing wheat with the relative and doubling chromosome number with colchicine to produce amphiploids. One of the best examples is *Triticale* the first cereal developed by human in a cross between wheat and rye, however, the hybrid was generated in Scotland in 1876, the first fertile hybrid was produced in 1938 (FEUILLET; LANGRIDGE; WAUGH, 2008; OETTLER, 2005). Nowadays, *Triticale* is widely used in the world, mainly for animal feed, since grain quality from bread wheat was not transferred.

Synthetic hexaploid wheat is another example developed to increase genetic variability, in order to rescue diversity already present in the wild forms, the first synthetic was developed by crossing the wheat ancestors *T. turgidum* (AABB) and *Ae. tauschii* (DD) (GILL; RAUPP, 1987). These synthetic lines are quite useful to

transfer desired genes to wheat because the synthetic hybrid will easily recombine due to the homology (CHAUDHARY; KAILA; RATHER, 2014).

Due to the genome composition of hexaploid wheat, it tolerates addition, substitution or deletion of a pair of chromosomes, single chromosomes, or part of it, thus addition, substitution, and deletions stocks can be developed to transfer traits from alien species. These lines are useful in pre-breeding programs, however, they often exhibit sterility, a repeated backcross of the amphiploid with the wheat parent can overcome this (FELDMAN; LEVY, 2005; SEARS, 1981).

Unfortunately, alien introgression always comes with the linkage drag, in general, the transfers should be as small as possible to exclude any flanking loci carrying genes with negative impact. Chromosome engineering, developed by Sears (1981), are cytogenetic manipulative approaches used to overcome the linkage drag and reduce the sizes of alien introgression. Induction of homeologous pairing is used to enhance the pairing between non-homologous chromosomes. The use of the 5B-deficient stocks (SEARS, 1981) or the recessive mutant *Ph1* (RILEY; CHAPMAN, 1958; ZHANG et al., 2004) have been used to promote homeologous pairing and recombination. And, as described before, the use of species suppressing *Ph1* is also an alternative to enhance recombination.

Ionizing Irradiation is used to induce centromeric breaks and spontaneous fusion between alien and wheat chromatin, however, the breaks are random leading to translocations among non-homeologous chromosomes resulting in duplications, or deficiencies in the progeny. Gametocidal induction is a method used transfer small alien segments and it is based on the capacity of gametocidal genes (*Gc*) to induce chromosome breaking in gametes where they are absent, ensuring that only gametes containing *Gc* genes are transmitted. However, ionizing radiation (SEARS; GUSTAFSON, 1993) and *Gc* (MASOUDI-NEJAD et al., 2002) have been used to transfer segments into wheat, in general, they seem do not compensate due to the genomic changes that often leads to a agronomically undesirable.

In an introgression program, the first step is to choose the wheat variety and the related species desired for the interspecific or intergeneric crosses, or choose the related species needed to make the synthetic. Then, if the goal is to produce addition, substitution or translocation lines, the lines should be screened for it, but if

the goal is to transfer small segments from the donor and keep most of the background from the chosen wheat, it is necessary to reduce the size of the segments, mainly due the linkage drag, and that can be accomplished by repeated backcrossing.

Even if a large scale phenotyping platform is available, and lines can be screened for the desirable traits, it is of huge importance to know what makes those lines genetically different, and where the differences are placed on, then evaluation of these introgressions is an important step in this kind of program.

2.8 Detection of introgressions

One of the major problems in an introgression-based breeding program is to detect these. Some approaches have been used in the past years. At the beginning chromosome recombination used to be detected using morphological markers and pairing behaviour during meiosis (SEARS, 1981). Then, differential staining started to be used mainly the C-banding, allowing the accurate identification of homeologous pairing in metaphase I and characterization of changes in chromosome structure based on the heterochromatin (FRIEBE; GILL, 1996; GILL; SEARS, 1988).

In situ hybridisation technique was first described by GALL and PARDUE (1969), and the procedure consists of using fluorescent probes to detect DNA sequences on the chromosomes. Fluorescence *in situ* hybridisation (FISH) is used to detect site-specific probes while genomic *in situ* hybridisation is used to detect whole genomes in a cell. Both techniques are used to detect alien chromosomes in interspecific hybrids.

However, the usage of *in situ* hybridisation to physically map alien introgression is limited to the size of the inserted segment, they must be large enough to be detected on the host chromosome (GUSTAFSON; DERA, 1989). The usage of GISH for the detection of alien chromosomes or recombinant segments in progenies among wheat and related species may be difficult due to the high degree of homology among them.

Besides these issues, cytogenetic techniques are laborious and time-consuming limiting the usage on a large scale, they are quite useful when combined

with molecular markers. Restriction fragment length polymorphisms (RFLP), amplified fragment length polymorphisms (AFLP), random amplified polymorphic DNA (RAPD), and mainly single sequence repeat (SSR) markers have been widely used to tag and map resistance genes, however, they are cost-ineffective for genotyping and laborious and time-consuming.

The development of next-generation sequencing technologies and high-throughput single nucleotide polymorphism (SNP) markers and corresponding SNP-arrays, has enabled faster more accurate detections of introgression from wild relatives into wheat (KING et al., 2017; TIWARI et al., 2014, 2015). That beginning with DArT (diversity array technology) (AKBARI et al., 2006), and following genotyping SNPs array platforms such as Illumina® 9K iSelect Beadchip Assay (CAVANAGH et al., 2013), Illumina® iSelect 90K SNP Assay (WANG et al., 2014) e Axiom® 820K SNP array (WINFIELD et al., 2016) were developed. Genotyping by sequencing (GBS) has been also used to generate maps with thousand *loci* (POLAND et al., 2012; SAINTENAC et al., 2013).

This high-throughput genotyping approach have been used to detect introgressions among wheat and relate species however they may be complemented by cytogenetic techniques, which allows the direct visualization of alien chromatin (GREWAL et al., 2018; SCHWARZACHER et al., 1992) and validate the SNPs that can be used to detect each of the wide species.

2.9 The use of *Triticum timopheevii* as a source of disease resistance

Triticum timopheevii Zhuk. is an allotetraploid wheat (genome formula A^tA^tGG), it belongs to the Timopheevii group. Its A^t genome has some similarity with the A from *T. turgidum* (AABB) and *T. aestivum* (AABBDD), according to strong evidence that they derived from the same ancestral *T. monoccoccum* L. ssp. *urartu* (ALLABY; BROWN, 2000; DVORAK et al., 1993; KILIAN et al., 2007b; TAKUMI et al., 1993). Although it does not have their B genome, (SEARS, 1969), it is suggested that its G genome is also originated from *Ae. speltoides* Tausch. or an ancestral close to it (DVORAK; ZHANG, 1990; MIYASHITA; MORI; TSUNEWAKI, 1994; OGIHARA; TSUNEWAKI, 1988; SASANUMA; MIYASHITA; TSUNEWAKI, 1996).

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3. Material and Methods

The experiment was conducted at The University of Nottingham / BBSRC Wheat Research Centre placed on the Sutton Bonington Campus in United Kingdom from 2014 to 2017.

3.1 Plant Material

Wheat lines carrying introgressions of chromosome segments of *T. timopheevii* in the BC_2 generation were pollinated with the wheat cv. TBIO Sinuelo. The BC_2 lines used as the donor parent were originally generated at the Nottingham/BBSRC Wheat Research Centre as part of the BBSRC funded WISP collaboration.

The Brazilian cultivar TBIO Sinuelo (protected by MAPA, nº 1085) used as the recurrent parent, was generously provided by its owner, Biotrigo Genética Ltd. This

genotype was chosen because it has been widely used in Brazil, due to its high adaptability and high yield. The crossing program is detailed in Figure 3.

3.2 Crossing program

3.2.1 Generation of BC_2 introgression lines accomplished by Nottingham/BBSRC Wheat Research Centre

In order to generate introgressions, mutant hexaploid wheat (*ph1/ph1*) either, Paragon or Chinese Spring varieties, were pollinated with *Triticum timopheevii* (accessions P95-99.1-1, 289752, 427414, 427998, 538512, 355452) to produce F_1 hybrids. Nottingham/BBSRC Wheat Research Centre obtained all accessions from the United States Department of Agriculture (USDA).

The F_1 hybrids were grown until maturity and backcrossed as the female with the euploid wheat parent, Paragon, to generate BC_1 populations. The BC_1 individuals were pollinated again with the wheat parent Paragon, to produce BC_2 populations.

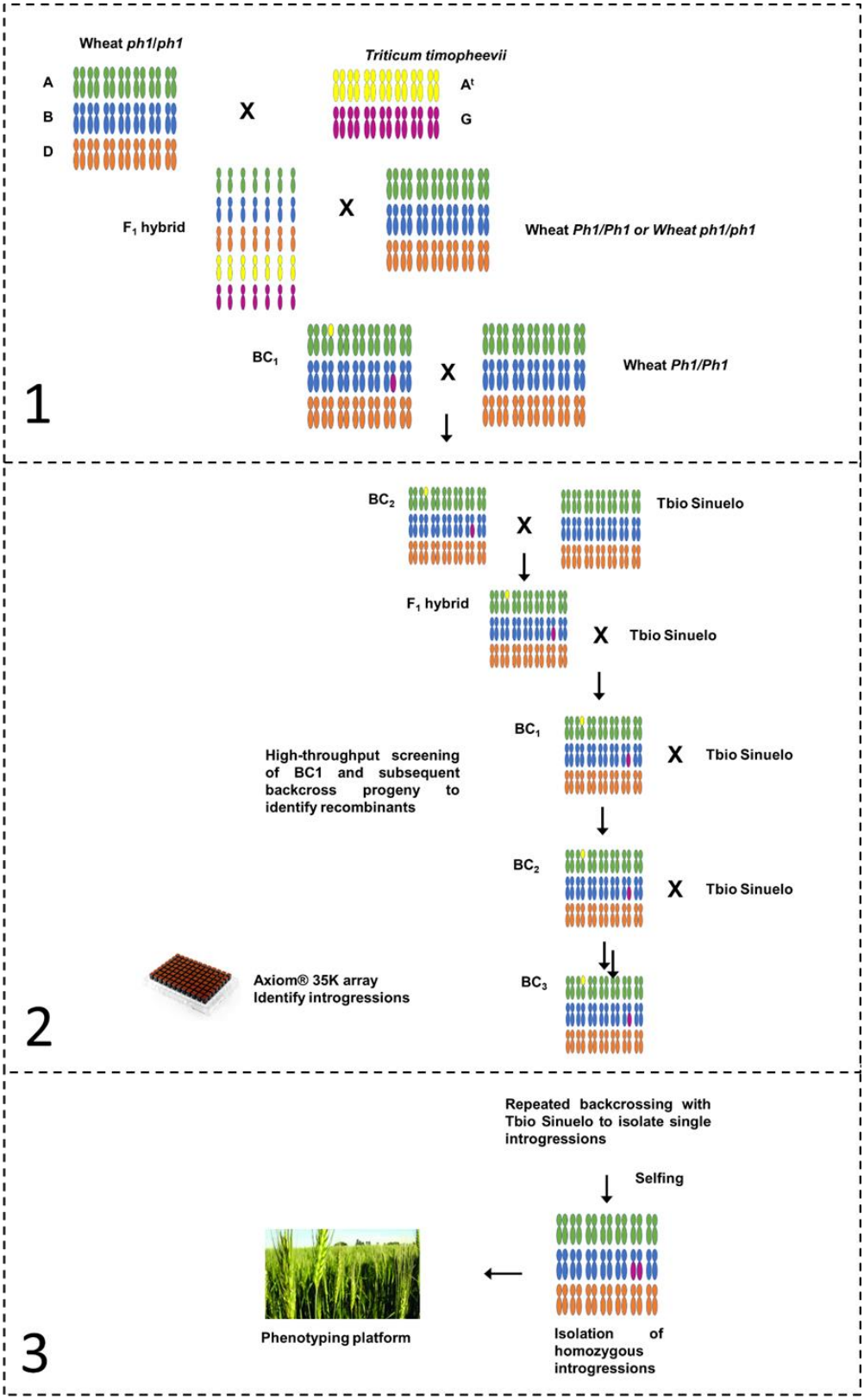

Figure 3 - Crossing scheme used to transfer introgressions from *T. timopheevii* into a Brazilian wheat cultivar (TBIO Sinuelo). 1. Steps accomplished by Nottingham/BBSRC WRC; 2. Steps accomplished by Brazilian group (CGF) at Nottingham. 3. Further steps to finish the program at UFPel.

Source: adapted from King et al. (2017).

3.2.2 Introduction of the Brazilian genotype

Thirty-two wheat lines carrying introgressions of chromosome segments of *T. timopheevii* in the BC_2 generation and their progenies were pollinated with the hexaploid wheat cv. TBIO Sinuelo to produce BC_3, BC_4, BC_5 and BC_6 populations. After the crossing between BC_2 introgression lines and the Brazilian wheat parent, the progeny was considered as a F_1 hybrid, and not as a BC_3 generation, so the BC_3, BC_4, BC_5 and BC_6 populations were named as BC_3 (F_1), BC_4 (BC_1), BC_5 (BC_2), and BC_6 (BC_3) respectively.

The decision of using both nomination was made because the Brazilian genotype was quite different from the previous wheat parents used, so more backcrosses would be needed to recover the desired genotype which is already adapted to the Brazilian environment, but the understanding of the real backcross generation is important for discussing some results obtained here.

3.3 Crossing procedure

The crossing experiment was conducted in a glasshouse at The University of Nottingham (UK). The first spike that emerged from every BC_2 plant was covered with a plastic bag to guarantee self-pollination to produce selfed seeds. Emasculation of spikelets from the BC_2 introgression lines was carried out when anthers were still immature, to avoid self-pollination and every emasculated head was covered with a plastic bag (Figure 4).

Florets were pollinated once stigmas seemed receptive, i.e. fluffy, about 3 days after emasculation and covered again with a plastic bag. To enhance the rate of fertilization, anthers from TBIO Sinuelo were collected and florets were pollinated one by one gently touching the ovary. Emasculation and pollination dates were recorded. Generally, four heads per plant were crossed. Backcrosses from further generations were accomplished the same way. When seeds were mature each head was harvested individually (figure 4). The heads were threshed manually. The seeds were kept in a cold chamber until the next crossing season.

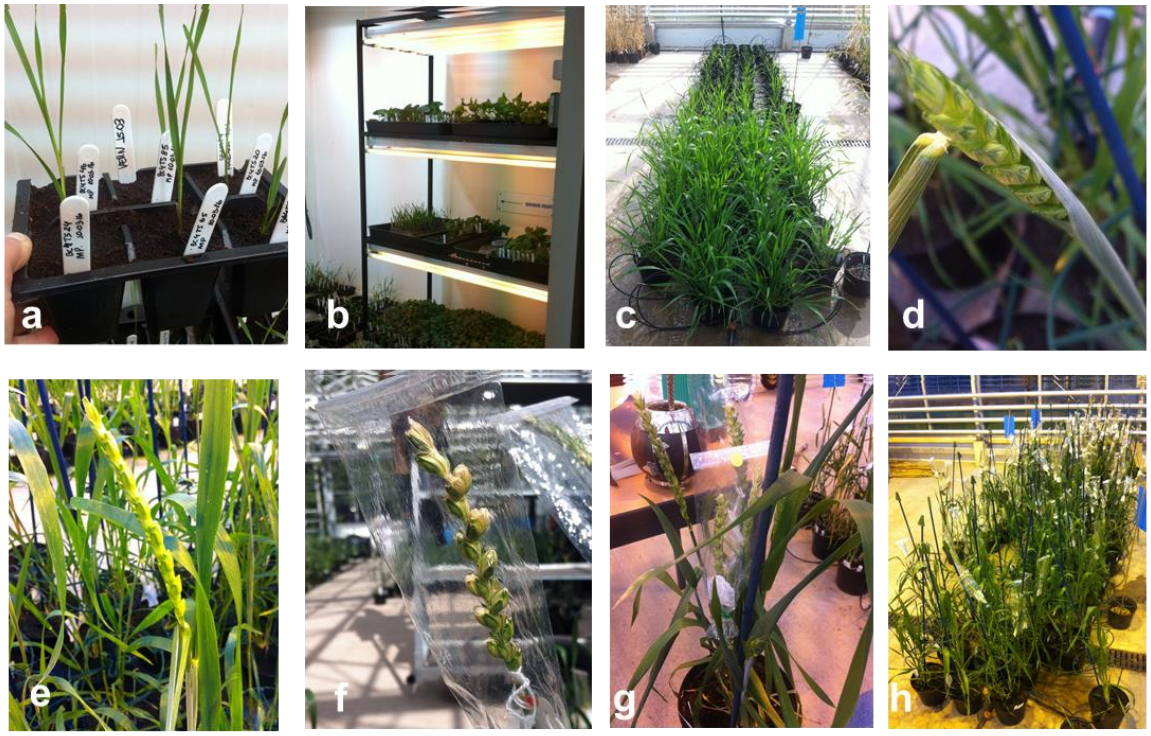

Figure 4 - Crossing procedure. a. Trays with seedling; b. vernalisation; c. TBIO Sinuelo plants at the glasshouse; d. head ready to emasculate; e. emasculated head; f. head with produced seeds; g. plant with self-fertilised and pollinated heads covered with plastic bag; h. crossing block.

3.4 Vernalisation and grown

Produced seeds were sown in Petri dishes and seedlings were transferred to trays with specific substrate (Figure 4). Trays were transferred to a vernalisation room when plants were grown enough to endure the cold (Figure 4). Backcrossed seedlings were vernalised at 6˚C from 4 to 8 weeks, depending on the generation. TBIO Sinuelo is a spring variety and it did not need vernalisation (Figure 4). At the end of the vernalisation treatment, the lines were potted individually in 2 litres pots with organic substrate and maintained in a glasshouse 16 hours of light at 20˚C and 8 hours of dark at 15˚C.

3.5 Selection of seeds

The first generations seeds were randomly selected for germination because at that time genotyping was not available. It was aimed to select as many plants as possible, considering the space available at the glasshouse and hands to deal with the crossing. For the later generations only seeds carrying introgressions were selected.

3.6 Seasons

BC_2 lines were pollinated with TBIO Sinuelo in January 2015 producing F_1 hybrids. 66 F_1 hybrids were selected at random and backcrossed with TBIO Sinuelo to produce the BC_1 individuals, two seasons were accomplished, one was made in august 2015 and an extra was made in January 2016 aiming to increase the number of BC_1 individuals. In June of 2016, 50 BC_1 individuals were selected and backcrossed again. December 2016, 28 BC_2 individuals were selected and backcrossed with TBIO Sinuelo producing BC_3 seeds.

3.7 Variables observed

Below are listed the variables observed in this program and how they were measured.

Seeds Sown: number of seeds sown

Germination rate (%): $\frac{\text{total number of seeds germinated}}{\text{total number of seed sown}} \times 100$

Crosses made: number of crosses made

Cross fertility: $\frac{\text{number of crosses producing seeds}}{\text{total number of crosses made}} \times 100$

Seed production: number of seeds produced from crosses

Seeds per cross: number of seed per crossing producing seeds

Self-fertilised seeds: number of self-fertilised seeds produced

3.8 DNA extraction

The lines were grown in organic substrate in 2 litres pots and maintained in a glasshouse 16 hours of light at 20˚C and 8 hours of dark at 15˚C. Leaf tissue was harvested from young plants, immediately frozen on liquid nitrogen and then stored at - 20 °C prior to DNA extraction.

DNAs from young leaves were isolated using extraction buffer [0.1 M Tris–HCl (pH 7.5), 0.05 M EDTA (pH 8.0), 1.25% SDS]. Samples were incubated at 65 °C for 1 h before being placed on ice and mixed with ice cold 6 M $NH_4C_2H_3O$ for 15 min. The samples were then spun down, the supernatant mixed with isopropanol to pellet the DNA and the isolated DNA was re-suspended in dH_2O.

DNA for Genomic *in situ* hibridisation were further purified with phenol/chloroform. DNA concentrations were checked and for the Affymetrix, sample dilutions were made in order to have DNA with 60 ng μL^{-1}, (desired concentration for the chip).

3.9 Genomic *in situ* hybridisation

The protocol for genomic in situ hybridisation (GISH) was as described in (KATO; LAMB; BIRCHLER, 2004; ZHANG et al., 2013) with the modifications of Grewal et al. (2018) and King et al. (2017).

Genomic DNA was isolated (see DNA extraction section) from young leaves of the three putative diploid progenitors of bread wheat, that is *T. urartu* (A genome), *Ae. speltoides* (B genome) and *Ae. tauschii* (D genome), and from *Ae. sharonensis* (related to B genome) and *T. timopheevii* (A^tA^tGG).

The genomic DNA of *T. timopheevii*, *Ae. speltoides*, *Ae. sharonensis* was labelled by nick translation with Chroma Tide Alexa Fluor 488-5-dUTP (Invitrogen; C11397; green). Genomic DNA of *T. urartu* was labelled with Alexa Fluor 594-5-dUTP (Invitrogen; C11400, red). Genomic DNA of *Ae. tauschii* and *T. aestivum* cv.

TBIO Sinuelo were fragmented to 300 - 500 bp in boiling water at 110 °C for 15 min, detailed protocol for preparation of probes is in Attachment A.

Seeds from every selected introgression line were germinated in Petri dishes for 2-3 days, when roots were 2 cm longer they were excised and treated with nitrous oxide gas at 10 bar for 2 h. Treated roots were fixed in 90% acetic acid for 10 min and then washed three times in water on ice. After the root collection seedlings were potted and send to vernalisation.

The root tip was dissected and digested in 20 µL of 1% pectolyase Y23 and 2% cellulase Onozuka R-10 (Yakult Pharmaceutical, Tokyo) solution for 50 min at 37 °C and then washed three times in 70% ethanol. The root tips were crushed against the tube wall in 70% ethanol, and the cells collected by centrifugation at 3000 x *g* for 1 min, briefly dried and then resuspended in 30 - 40 µL of 100% acetic acid before being placed on ice.

The cell suspension was dropped onto glass slides (6 - 7 µL per slide) in a moist box and dried slowly under cover. Chromosome spreads were checked in microscope and selected slides were initially probed for single GISH using labelled genomic DNA of *T. timopheevii* (100 ng) and fragmented genomic DNA of TBIO Sinuelo (3000 ng) as blocker to detect the *T. timopheevii* segments. Probe to block was in a ratio of 1:30 (the hybridisation solution was made up to 10 µL with 2 x SSC in 1 x TE).

A multicolour GISH was also performed as an attempt to detect introgressions. The slides were probed with labelled DNAs (*T. urartu*, *Ae. spelotides*, *Ae. sharonensis*) and fragmented DNA of *Ae. tauschii* as blocker in the ratio 1:30. The different hibridisation solutions are presented in table 1.

All slides were counterstained with DAPI and analysed using a Leica DM5500B epifluorescence microscope (Leica Microsystems, Wetzlar, Germany) with filters for DAPI (blue), Alexa Fluor 488 (green) and Alexa Fluor 594 (red). Photographs were taken using a Leica DFC 350FX digital camera.

Table 1 – Different hybridisation solutions used for single and multi-colour GISH technique in spreads of TBIO Sinuelo, *T. timophevii* and BC lines using different genomes as probe and *Ae. tauschii* used as blocker

Spread	Probe				Blocker
	µL				
	T. timopheevii	*T. urartu*	*Ae. speltoides*	*Ae. sharonensis*	*Ae. tauschii*
TBIO Sinuelo	1.0	-	-	-	9.0
TBIO Sinuelo	1.0	1.0	1.0	-	8.0
T. timopheevii	-	1.0	1.0	-	8.0
T. timopheevii	-	1.0	-	1.0	8.0
BC_3 (F_1) line	-	1.0	1.0	-	8.0
BC_3 (F_1) line	-	1.5	-	1.0	7.5

3.10 Identification of introgressions via an Affymetrix SNP array

To detect introgressed chromosomes and chromosome segments from *Triticum timopheevii* into wheat an array of circa 35 K SNPs (Axiom® Wheat-Relative Genotyping Array) showing polymorphism between ten wild relatives, including *T. timopheevii*, and some wheat varieties was used. This array was developed by the Nottingham/BBSRC Wheat Research Centre (KING et al., 2017; WINFIELD et al., 2016) and it is part of the Axiom_ 820K SNP array, (WILKINSON et al., 2012, 2016) which data set is available from www.cerealsdb.uk.net.

3.11 Genotyping

The equipment, software, procedures and criteria detailed bellow was as described in King et al. (2017).

The Axiom-Wheat-RelativeGenotyping Array was used to genotype 136 samples from BC_1, BC_2, BC_3 (F_1), BC_4 (BC_1) generations, using the Affymetrix GeneTitan_system according to the procedure described by Affymetrix (Axiom_ 2.0 Assay Manual Workflow User Guide Rev3). Allele calling was carried out using the Affymetrix proprietary software packages Affymetrix Power Tools (APT) and

SNPolisherTM (http://www.affymetrix.com/estore/partners_programs/programs/developer/tools/devnettools.affx).

A custom software pipeline ADAP (Axiom Data Analysis Pipeline) was used, following the AxiomBest Practices Genotyping Workflow (http://media.affymetrix.com/support/downloads/manuals/axiom_genotyping_solutio_analysis_guide.pdf). A variant call rate threshold of 80% was used to account for the lower call rates typically obtained from hybridising wheat relatives and progenitors to the array.

The aptprobeset-genotype program within Affymetrix Power Tools determines genotype calls from Affymetrix SNP microarrays. Following this, the SNPolisher R package calculates SNP performance metrics, such as call rate, cluster separation and deviation from expected cluster position. It then classifies the SNPs into performance categories. Polymorphic high resolution (PHR) is a category where calls which were considered to give good cluster resolution with at least two examples of the minor alleles for genetic mapping purposes. Only the PHR SNPs were used as they provide good cluster resolution where each SNP essentially behaves like a diploid.

3.12 Genetic Mapping of *T. timopheevii* chromosomes

Individuals from a backcross population between *T. aestivum* and *T. timopheevii* were genotyped with the Axiom® Wheat-Relative Genotyping Array. Along with duplicates of the parental lines, 136 lines comprising BC_1, BC_2, BC_3 (F_1), BC_4 (BC_1) populations of *T. aestivum*/*T. timopheevii* were genotyped. As stated above, only the PHR SNP markers were used for genetic mapping. SNP markers which showed heterozygous calls for either *T. timopheevii* or wheat parent(s), no polymorphism between the wheat parents and *T. timopheevii* and/or no calls for either parent(s) were removed using FlapjackTM v.1.14.09.24 (MILNE et al., 2010).

The resulting markers were sorted into linkage groups in JoinMap 4.1 (VAN OOIJEN, 2011) with a LOD score of 40 and a recombination frequency threshold of 0.1 using the Haldane mapping function (HALDANE, 1919). All markers that did not show any heterozygous call or were unlinked were ignored and only the highest

ranking linkage groups with more than 30 markers were selected for map construction.

These were exported and assigned to chromosomes using information from the AxiomWheat HD Genotyping Array (WINFIELD et al., 2016). Where chromosomes were split into multiple linkage groups. Erroneous markers that had more than 20% missing data or showed a unique pattern of segregation that was either not observed in the previous backcross generation or not consistent with the recombination of neighbouring markers in the group, in different samples, were also removed.

The markers of each chromosome were aligned with the wheat genome from the IWGSC wheat survey sequence (https://wheat-urgi.versailles.inra.fr/morgoth/Tools/BLAST), and they were reordered according to the marker position at the wheat genome. A^t genome was aligned to wheat A, and G was aligned to wheat B genome. Final map reordering was conducted with JoinMap 4.1 (VAN OOIJEN, 2011) and genetic maps produced through MapChart 2.3 (VOORRIPS, 2002). In some cases, physical map information was employed to order loci. Graphical genotype visualization was performed using Graphical GenoTypes 2.0 (VAN BERLOO, 2008).

3.13 Comparative analysis

Synteny analysis was carried out using sequence information of the markers located on the present map of *T. timopheevii*. The sequences of the mapped markers were compared using BLAST (e-value cut-off of $1e^{-05}$) against the wheat genome (IWGS) to obtain the orthologous map positions of the top hits in the A, B and D genomes of wheat. For the synteny observation the software Circos v.0.69-6 (KRZYWINSKI, 2009) was used. To generate the figures, cM distances on the linkage groups of the present map of *T. timophevii* were scaled up by a factor of 10^6 to match similar base pair lengths of the chromosomes of the wheat genome.

4. Results and discussion

4.1 Generation of germplasm

A total of 548 crosses were made between brazilian wheat (cv. TBIO Sinuelo) and *T. timopheevii* (via BC_2 introgression lines) in all backcross generations, 4,579 seeds were obtained from crossed ears, and additionally 3,890 self-fertilised seeds were produced. The germination rate, number of crosses made, cross fertility rate, seed production, number of seeds per cross and number of self-fertilised seeds are detailed by generation in table 2.

Table 2 - Number of seeds produced and germinated in relation to the number of crosses carried out and number of self-fertilised seeds for each generation of the introgression program for *T. timopheevii* and Brazilian wheat (cv. TBIO Sinuelo)

	Seeds Sown (n°)	Germination rate (%)	Crosses made (n°)	Cross fertility (%)	Seed production (n°)	Seeds per cross	Self-fertilised seeds (n°)
BC_2 *TTIL* x Sinuelo	-	-	67	100	722	10.78	-
F_1	73	90	275	87.6	2,164	7.87	1,088
BC_1	55	91	178	87.1	1,609	9.04	2,781
BC_2	27	89	28	75	84	3	21
Total	155	-	548	-	4,579	-	3,890

TTIL: *Triticum timopheevii* introgression lines; Sinuelo: TBIO Sinuelo

Thirty-two introgression lines in BC_2 generation were pollinated with TBIO Sinuelo, 67 crosses were made and 722 F_1 seeds were obtained from them. 66 F_1 plants were selected at random and backcrossed with TBIO Sinuelo, resulting in a total of 2,164 BC_1 seeds from 275 crosses. 50 BC_1 individuals were selected and backcrossed again, resulting in a total of 1,609 BC_2 seeds from 178 crosses. 24 BC_2 plants were also backcrossed resulting in a total of 84 BC_3 seeds from 28 crosses.

All the generations showed a high rate of germination of over 90% for F_1 and BC_1 generations and 89% for BC_2, although germination rate was not a 100%, all the germinated seedlings survived until maturity. In order to estimate the cross fertility of

all backcross generations, the number of crossed ears that produced seeds were recorded, and high fertility levels of 87.6%, 87% and 75% for; F_1, BC_1 and BC_2 generations, respectively, were observed. High fertility level means that most of the crosses produced seeds.

Even with the high fertility for BC_2 plants the value was lower than the other generations, this is an unexpected result since fertility rate is expected to increase over the generations. In addition, the proportion of self-fertilised seeds per plant producing seed for this season was really low, 0.85:1 comparing to 16.48:1 (F_1) and 55.62:1 (BC_1), it indicates that environmental issues could have affected seed production in that generation and is most likely since a different glasshouse was used to grow the BC_2 plants.

T. timopheevii is an important source of genes for resistance to disease (LEONOVA et al., 2011) and up to date twelve genes conferring resistance have already been transferred into wheat (MCINTOSH et al., 2013). A genome transference approach was used via BC_2 lines carrying chromosome additions, substitutions and introgressed segments. An elite Brazilian wheat genotype, cultivar TBIO Sinuelo was used as recurrent parent due to its wide grain yield and adaptability.

Gene introgression occurs when at meiosis related chromosomes from the two parental species recombine resulting in the generation of inter-specific recombinant chromosomes, which is the F_1 hybrid. The recombinant chromosomes are then transmitted to the next generation through the gametes (REYNOLDS et al., 2012). In this work, the thirty-two BC_2 lines used to transfer segments from *T. timopheevvii* came from fifteen BC_1 lines carrying segments previously developed by WRC (data not shown). Thus the number of introgressions that could be generated in this work was limited to the 15 female F_1 gametes which originated the 15 BC_1 plants, if no further recombination occurred in later generations, so that would be the gametes present in BC_1, BC_2 and BC_3 generations.

The repeated backcrossing of the F_1 hybrid to the wheat genotype will result in the generation of lines which carry the majority of wheat genome but also carry one or more chromosome segments from the other parental species (REYNOLDS et al., 2012). F_1 hybrids between *T. timopheevii* and wheat are self-sterile, and self-fertility

is usually restored after a couple of backcrosses (JARVE; JAKOBSON, 2002). The F_1 hybrids obtained by crossing the BC_2 introgression lines (see material and methods) and the Brazilian wheat were self-fertile, which means they produced self-fertilised seeds, that occurred because they are actually BC_3 lines considering wheat background, i.e., the backcrossing strategy has already restored its fertility.

Comparing the F_1 (BC_3) generation produced in this work using *T. timopheevii* as the wild relative with the BC_3 generation produced in other programs using the same approach (GREWAL et al., 2018; KING et al., 2017, 2018; VENSKE, 2017), *T. timopheevii* shows a better potential to be used in such programs. Cross fertility and number of seeds per cross were considerably high and quite similar with wheat/*Thinopyrum bessarabicum* (GREWAL et al., 2018) and wheat/*Amblyopyrum muticum* (KING et al., 2018) BC_3 lines.

4.2 Genomic *in situ* hybridisation

To confirm SNP analysis, genotyped individuals were selected and analysed via single and multicolour GISH. Many attempts were made trying to identify introgressions but a clear signal was not detected in most of the chromosome spreads and this will be discussed later in this section. Together with the wheat/*T. timopheevii* lines, metaphase spreads of parental lines, TBIO Sinuelo and *T. timopheevii,* were probed to visualise the genome constitution at the whole chromosome level.

The multicolour GISH technique enabled the identification of the genome three genomes of wheat (A genome - pink, B genome - green and D genome - purple) and the arrows in figure 5 show the already identified 4A/5A/7B wheat translocation (LIU et al., 1992; NARANJO et al., 1987). This technique also helped identifying the two genomes of *T. timopheevii* (A^t genome - red/pink and G - genome green) and, the arrows in figure 6b show a A^t/G translocation which could be $6A^tS$/1GS translocations previously reported (BROWN-GUEDIRA et al., 1996). Although GISH has been widely used to identify alien chromosomes in interspecific hybrids i.e. wheat/*Ae. speltoides* (KING et al., 2018; VENSKE, 2017), wheat/*Am. muticum* (KING et al., 2017) and wheat/*Th. bessarabicum* (GREWAL et al., 2018), for *T. timopheevii* the

attempt used in this study did not work well. Figure 7 shows on the left side a spread of the wheat cv. TBIO Sinuelo probed with labelled *T. timopheevii* genomic DNA (green) and blocked with *Ae. tauschii* (blue), of the 42 wheat chromosomes, 28 are presenting green signals. On the right side there is a spread of wheat/*T. timopheevii* line probed with labelled genomic DNA of *T. timopheevii* (green) in this case the genomic DNA of wheat cv. TBIO Sinuelo was used as blocker (blue), no green signals are observed.

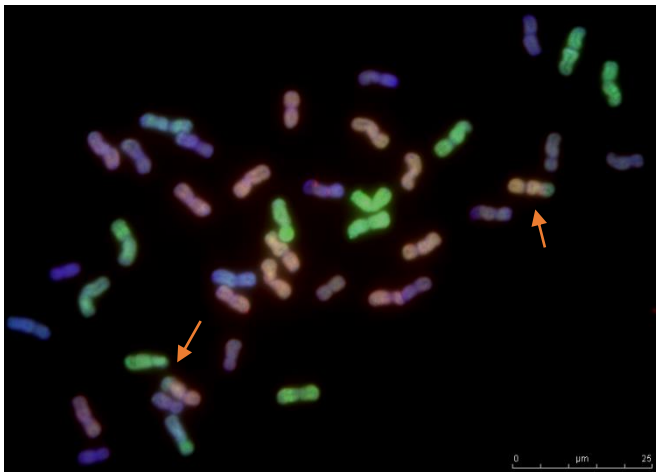

Figure 5 - Genomic *in situ* hybridisation images show two metaphase spread of the wheat cv. TBIO Sinuelo probed with labelled genomic DNA of *T. urartu* (pink), *Ae. speltoides* (green). *Ae. tauschii* genomic DNA was used as blocker (purple).

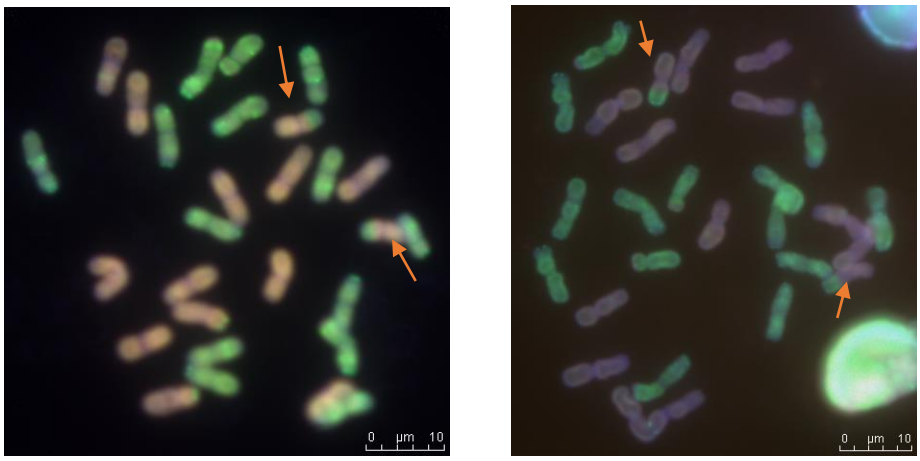

Figure 6 - Genomic *in situ* hybridisation image shows two metaphase spreads of the *T. timopheevii*. The left was probed with labelled genomic DNA of *T. urartu* (red) and *Ae. sharonensis* (green). The right was probed with labelled genomic DNA of *T. urartu* (pink) and *Ae. speltoides* (green).

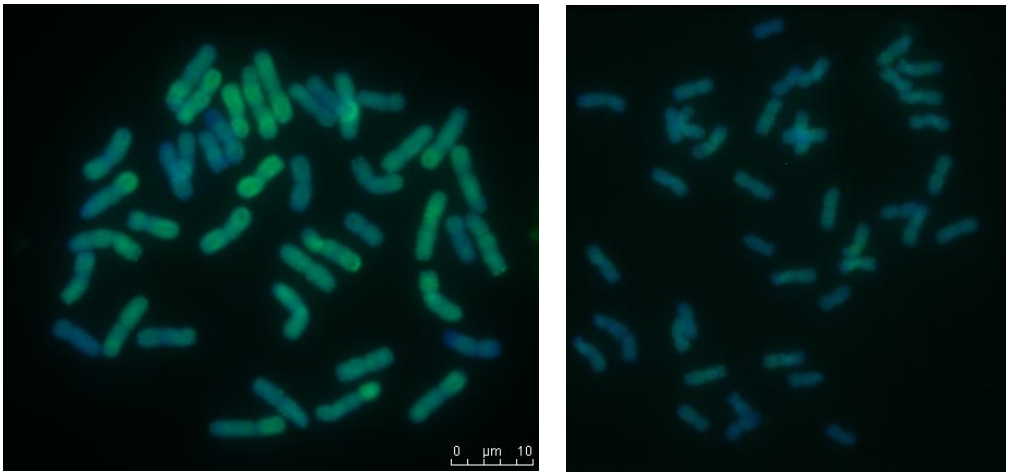

Figure 7 - Genomic *in situ* hybridisation images show: on the left wheat cv. TBIO Sinuelo spread probed with labelled genomic DNA of *T. timopheevii* (green). *Ae. tauschii* genomic DNA was used as blocker (blue). On the right wheat/ *T. timopheevii* line probed with labelled genomic DNA of *Timopheevii* (green). TBIO Sinuelo genomic DNA was used as blocker (blue)

The presence of a signal in the wheat spread when *T. timopheevii* genomic DNA is used as the probe and *Ae. tauschii* as the blocker, and a absence signal in *T. timopheevii* with the same probe but wheat as the blocker, indicates that there is a high degree of shared sequence homology between the two, making it difficult to identify alien chromosomes and recombinant segments in interspecific hybrids in the recipient progenies using the GISH approach.

To date, there are no published data describing the use of GISH to detect alien chromosomes in the recipient progenies between *T. aestivum* and *T. timopheevii*. N and C-banding (BADAEVA et al., 1991; BROWN-GUEDIRA et al., 1996; MAESTRA; NARANJO, 1999) or Fluorescent in situ hybridisation (DEVI, 2017) have been used.

However, mc-GISH could be effective in detecting introgressions in wheat/*T. timopheevii* hybrids using *Ae. tauschii* as blocking if the recombination takes place with the D genome of wheat. Figure 8, shows one spread of wheat/*T. timopheevii* line in BC_3 (F_1) generation, and the arrow shows one recombinant event, the green signal at the D genome. The green probe represents the *Ae. speltoides* genome, the closest related species to the B genome of wheat, and the G genome of *T. timopheevii*. Figure 5, shows a TBIO Sinuelo spread and it does not present that event in any of its D chromosomes, thus the segment may have come from *T.*

timopheevii. SNP analysis of the same individual (Figure 8) showed a putative introgression coming from the linkage group (LG) $5A^t$, therefore, the visualised signals should be red, since *T. urartu* (donor of the A genome present in both species), was labelled with a red colour.

Substitution as a rule, happened among the related genomes A-A^t and B-G, and it can be explained because A-A^t and B-G genome chromosomes are less genetically divergent and pair at meiosis of F_1 interspecific hybrids (GILL; CHEN, 1987; GILL; SEARS, 1988), but exceptions occur and substitutions of the G for D genome chromosome has previously reported (BADAEVA et al., 1991).

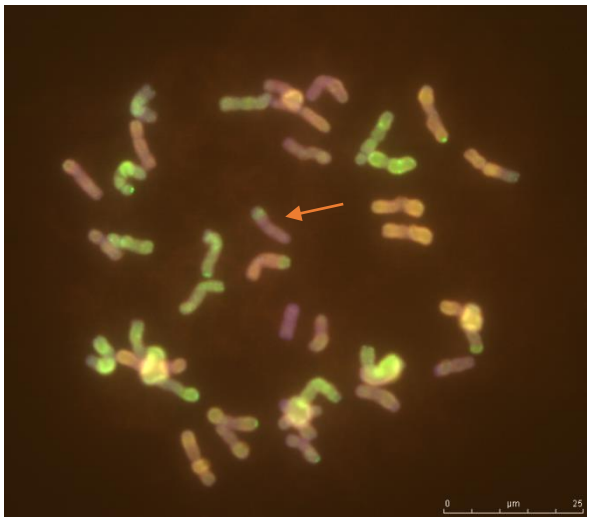

Figure 8 - Genomic *in situ* hybridisation image shows a metaphase spread of the wheat/*T.timopheevii* line probed with labelled genomic DNA of *T. urartu* (pink), *Ae. speltoides* (green). *Ae. tauschii* genomic DNA was used as blocker (purple).

The putative introgression from the $5A^t$ LG, probably followed that rule, and happened to the related A genome, and as discussed before, it could not be detected via GISH analysis due to the high homology between the genomes, but a segment related to the *Ae. speltoides* (B/G) was detected at the D genome, either that segment came from G genome of *T. timopheevii* and the markers corresponding to that region were not available or excluded during the SNP analysis, or they came from a recombinant event between B and D genome of wheat.

Slides of metaphase spreads were prepared for every BC line used for crossing. After the genotyping analysis, some lines having whole chromosome substitution were submitted to the mc-GISH approach, they were probed with different labels and with different probe/blocking proportions and only one showed signals of introgressions in the D genome.

4.3 Genotyping and genetic mapping

Of the 35 thousand SNPs on the Axiom® Wheat-Relative Genotyping Array, 5,334 (15.2%) were considered polymorphic between wheat and *T. timopheevii* with high resolution (PHR). As stated before, only the PHR SNPs were used to create the genetic map as these are considered to be optimum quality. They were used to construct the genetic map allowing the formation of the *T. timopheevii* linkage groups (Table 3, Figures 9 and 10).

PHR calls were exported to Flapjack ™ v.1.14.09.24 (MILNE et al., 2010) and markers showing heterozygous call for either parents, no polymorphism between the wheat parents and *T. timopheevii* and no calls for either parents were removed. Of the 5,334 PHR markers, 748 were removed and the left over 4,586 were exported to JoinMap® 4.1 (VAN OOIJEN, 2011) for map construction and it resulted in fourteen chromosomes representing *T. timopheevii.*

In total 1,432 SNP markers were mapped in the population across all the 14 chromosomes, the LOD scores used varied from 13 to 25. Table 3 shows that 421 SNPs were assigned to A^t genome and 1,011 to G genome. The cM lengths of A^t linkage group 1 – 7 were 105, 108.3, 94.7, 89.7, 162, 125 and 57.5, respectively. The total length of A^t frame was 742.2 cM and the average length by chromosome was 105 cM. For the G linkage group, the cM length of 1 – 7 were 58.5, 112.6, 44.6, 103.6, 94.2, 84.1 and 59.5, respectively. The total length of the G frame was 557 cM with an average 84.08 cM per chromosome.

Table 3 - Number of polymorphic SNPs between *T. timopheevii* and Brazilian hexaploid wheat based on the Affymetrix 35K array. Relative Genotyping Array for each linkage group of the A^t and G genomes and final number of SNP markers mapped onto the physical map of the *T. timopheevii* obtained through Poly High Resolution (PHR) calling

	Linkage group								
	$1A^t$	$2A^t$	$3A^t$	$4A^t$	$5A^t$	$6A^t$	$7A^t$	Total	Average
No. of SNPs	74	72	72	31	43	65	64	421	65
Lengh (cM)	105	108.3	94.7	89.7	162	125.0	57.5	742.2	105
LOD score	15	13	21	22	15	18	17	-	17
	Linkage group								
	1G	2G	3G	4G	5G	6G	7G	Total	Average
No. of SNPs	72	256	108	121	279	22	153	1,011	121
Lengh (cM)	58.5	112.6	44.6	103.6	94.2	84.1	59.5	557.0	84.08
LOD score	15	20	22	13	19	16	25	-	19

The level of interspecific recombination detected by the genetic mapping was such that it was possible to assemble the fourteen linkage groups of *T. timopheevii*. SNP markers were well distributed over 7 linkage groups of the both genomes, the marker names and cM distances for each 14 linkage groups are shown in Appendix A.

The *T. timopheevii* genetic map constructed here, allowed the characterization of putative introgressions. In order to have enough number of individuals, the mapping populations consisted of different backcross generations, thus, the cM distances of the linkage groups, should be treated with caution since it is not a proper mapping population, wild species have some mechanism which favoured the transmissions of some chromosomes causing mendelian distortions affecting and the statistical analysis used to detect the linkage (ENDO, 1990; NIRANJANA, 2017).

Comprising all generations, including the ones previously developed for Nottingham Wheat Research Centre, 631 putative introgressions were found via genotyping using genetic linkage map of *T. timopheevii*. However, 316 putative introgressions were found in the generations backcrossed to TBIO Sinuelo (Table 4). All the linkage groups presented putative segments, only one plant in BC_4 (BC_1) generation did not show any segment. SNP analysis revealed that 10 plants presented putative single introgression transferred to the Brazilian genotype, and they were originated from $4A^t$, 1G, 2G, 5G, 6G and 7G linkage groups.

Table 4 - Number of putative introgressed segments from *T. timopheevii* transferred to the Brazilian genotype TBIO Sinuelo, in BC_3 (F_1) and BC_4 (BC_1) plants detected by SNP genotyping. The *T. timopheevii* have been assigned to linkage groups via the comparative analysis of the SNPs with wheat

Family	Generation	Code	No segm.	No. whole segm.	Origin of the whole segments			
1	BC_3 (F_1)	1A	3					
	BC_3 (F_1)	1B	6	3	1G	$3A^t$	$4A^t$	
	BC_3 (F_1)	2A	7	1	1G			
	BC_3 (F_1)	2B	7	3	1G	$3A^t$	$4A^t$	
	BC_3 (F_1)	3	5					
	BC_4 (BC_1)	1	3					
	BC_4 (BC_1)	123	4					
2	BC_3 (F_1)	4	4					
	BC_4 (BC_1)	2	1					
	BC_3 (F_1)	116A	6	2	$3A^t$	$4A^t$		
	BC_3 (F_1)	116B	4	1	5G			
	BC_3 (F_1)	5A	3	1	$7A^t$			
	BC_3 (F_1)	5B	5	2	$3A^t$	$7A^t$		
	BC_3 (F_1)	6	4	1	$7A^t$			
	BC_4 (BC_1)	5	4	1	$7A^t$			
	BC_4 (BC_1)	6	3					
	BC_3 (F_1)	6B	2	1	$3A^t$			
3	BC_3 (F_1)	7	6	2	6G	7G		
	BC_4 (BC_1)	9	3					
	BC_4 (BC_1)	10	4	1	6G			
	BC_4 (BC_1)	14	1					
	BC_3 (F_1)	7C	5	4	$3A^t$	$4A^t$	6G	7G
	BC_3 (F_1)	9	3	1	2G			
	BC_4 (BC_1)	17	1	1	2G			
	BC_4 (BC_1)	18	2					
	F_1 (BC_3)	9B	4					
	F_1 (BC_3)	10	3					
	BC_4 (BC_1)	20	3					
	BC_4 (BC_1)	22	3					
	BC_3 (F_1)	11	2					
	BC_4 (BC_1)	24	2					
	BC_4 (BC_1)	25	1					
	BC_3 (F_1)	11B	1	1	7G			
	BC_3 (F_1)	12A	3	1	7G			

	BC_4 (BC_1)	34	1					
	BC_4 (BC_1)	35	2	1	7G			
	BC_3 (F_1)	12B	2	1	6G			
	BC_4 (BC_1)	39	2	1	6G			
	BC_4 (BC_1)	42	2	1	6G			
4	BC_3 (F_1)	13	11	4	$1A^t$	1G	4G	7G
	BC_4 (BC_1)	46	8	3	$1A^t$	4G	7G	
	BC_3 (F_1)	14	7					
	BC_4 (BC_1)	50	4					
	BC_3 (F_1)	15A	6	3	$2A^t$	$3A^t$	$4A^t$	
	BC_3 (F_1)	15B	6	2	$3A^t$	$4A^t$		
	BC_3 (F_1)	66	4	2	$3A^t$	$4A^t$		
	BC_4 (BC_1)	54	2	1	$3A^t$			
5	BC_3 (F_1)	16	4	2	$4A^t$	$5A^t$		
	BC_3 (F_1)	17A	5	1	$4A^t$			
	BC_3 (F_1)	17B	3					
	BC_4 (BC_1)	56	2					
	BC_4 (BC_1)	60	2					
	BC_3 (F_1)	17C	4	2	3G	7G		
	BC_3 (F_1)	17D	3	1	3G			
	BC_3 (F_1)	18	5	2	3G	7G		
	BC_1(BC_4)	61	5	2	3G	7G		
	BC_1(BC_4)	65	2	1	7G			
	BC_3 (F_1)	18B	4	1	7G			
	BC_3 (F_1)	19	2	1	3G			
	BC_4 (BC_1)	71	1					
	BC_3 (F_1)	20	4	1	2G			
	BC_4 (BC_1)	74	4					
	BC_3 (F_1)	21	2					
	BC_4 (BC_1)	75	1					
	BC_3 (F_1)	21B	4					
	BC_4 (BC_1)	119	2					
6	BC_3 (F_1)	24	2	1	2G			
	BC_1(BC_4)	81	2	1	2G			
	BC_4 (BC_1)	83	1	1	2G			
	BC_3 (F_1)	26A	4	3	2G	$7A^t$	7G	
	BC_3 (F_1)	28	3					
	BC_4 (BC_1)	85	0					

	BC_4 (BC_1)	87	3	1	4G		
	BC_1(BC_4)	88	2				
	BC_4 (BC_1)	28B	2	1	5A^t		
	BC_3 (F_1)	30A	5	3	1A^t	2G	5G
	BC_3 (F_1)	31A	2	2	1A^t	2G	
7	BC_3 (F_1)	32A	2	1	2G		
	BC_3 (F_1)	33A	1	1	2G		
	BC_3 (F_1)	34A	5	1	3G		
8	BC_3 (F_1)	36A	3	1	2G		
	BC_3 (F_1)	69A	3				
9	BC_3 (F_1)	72A	4				
	BC_3 (F_1)	73A	4	2	1A^t	5A^t	
10	BC_3 (F_1)	74A	6	1	4A^t		
	BC_3 (F_1)	75A	2				
11	BC_3 (F_1)	77A	3	1	1A^t		
	BC_3 (F_1)	78A	2				
12	BC_3 (F_1)	79A	4	2	2A^t	2G	
13	BC_3 (F_1)	82A	4	1	2G		
	BC_3 (F_1)	86A	5	3	3G	4A^t	6G
	BC_3 (F_1)	91A					
14	BC_3 (F_1)	91B	2				
	BC_3 (F_1)	92A	2				
	BC_4 (BC_1)	48	4	2	1A^t	1G	
	Total		316	89			

The problems to exploit wild relatives has been the absence of appropriate high-throughput technologies to screen for, and specifically identify, introgression events (KING et al., 2017). The Affymetrix wheat/wild relative array used here enabled the identification and characterization of genome-wide putative introgressions of various sizes. The ability to detect these introgressions is a direct result of using the SNP array, that is large numbers of markers to detect genomewide introgressions have not been previously available.

The WRC have successfully used Affymetrix Array to detec introgression from wild relatives of wheat within a wheat background. The advantage of this approach is that we are able to use the one dedicated array to identify and characterize introgressions from all ten of the wild relative species we are currently working with, without the need for a reference genome (GREWAL et al., 2018; KING et al., 2017, 2018). To date, wheat Research Centre have screened approximately 3,800 individual genotypes via the array. The WRC from UoN is converting some the SNP markers from this Array into KASP markers thus it will allow us to track individual introgressions quickly and cost effectively, through the process of backcrossing and selfing through marker-assisted selection.

SNP analysis during genetic mapping should be validated with cytological approach, even though as stated before, GISH analysis was not efficient at detecting introgression in *T. aestivum*/*T. timopheevii* hybrids. The high homology between the A^tG genomes of *T. timopheevii* and AB genomes of hexaploid wheat affects the specificity of the probes and the blocking DNA and thus, prevents any meaningful cytological detection (SCHNEIDER; MOLNÁR; MOLNÁR-LÁNG, 2008).

Devi (2017) also used Axiom® Wheat-Relative Genotyping Array for genotyping from *T. aestivum*/*T. timopheevii* hybrids, and she was able to validate SNP analysis from 10 lines using a combination of DNA probes with FISH technique. Afa-family and pSc119.2 were used to identify A^t and G chromosomes, respectively (MIKÓ et al., 2014). Unfortunately, to detect small segments the introgression should be located in the region presenting signals, therefore only lines having whole introgressed segments were used for FISH analysis.

From 347 genotyped individuals, (DEVI, 2017), only validated 10 lines using FISH. Badaeva et al. (1991) analysed 35 *T. aestivum*/*T. timopheevii* hybrids with C-banding. C-banding, N-banding or FISH techniques, as discussed in the introduction section, allowing us to identify single introgressions, even though the cytological analysis are technically demanding and time consuming and they are not cost effective for using on a large-scale.

This program has generated 4,579 seeds having a Brazilian genotype from the cultivar TBIO Sinuelo in their background, 96 individuals were genotyped and they present *T. timopheevii* segments. A number of molecular markers were identified and

they can be used to characterise *T. aestivum*/*T. timopheevii* introgression lines and maybe translocation lines involving Timopheevii wheats. The linkage map generated here allows the identification of 316 putative introgressions, to be characterised and available to be used in wheat breeding programs in Brazil.

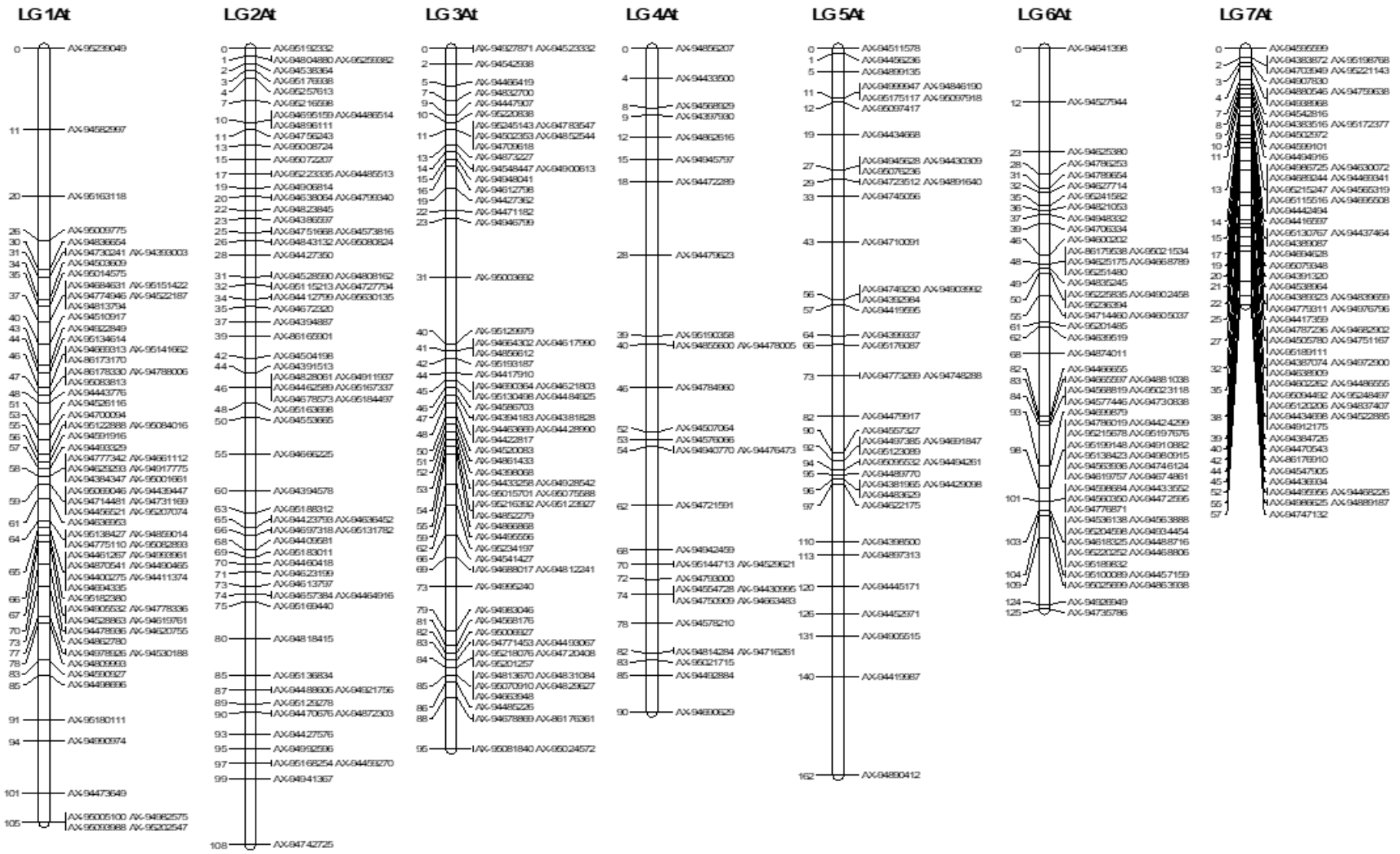

Figure 9 - Genetic linkage map of the A^t genome of *T. timopheevii*. SNP marker names and cM distances for each of the seven linkage groups are also shown in appendix A.

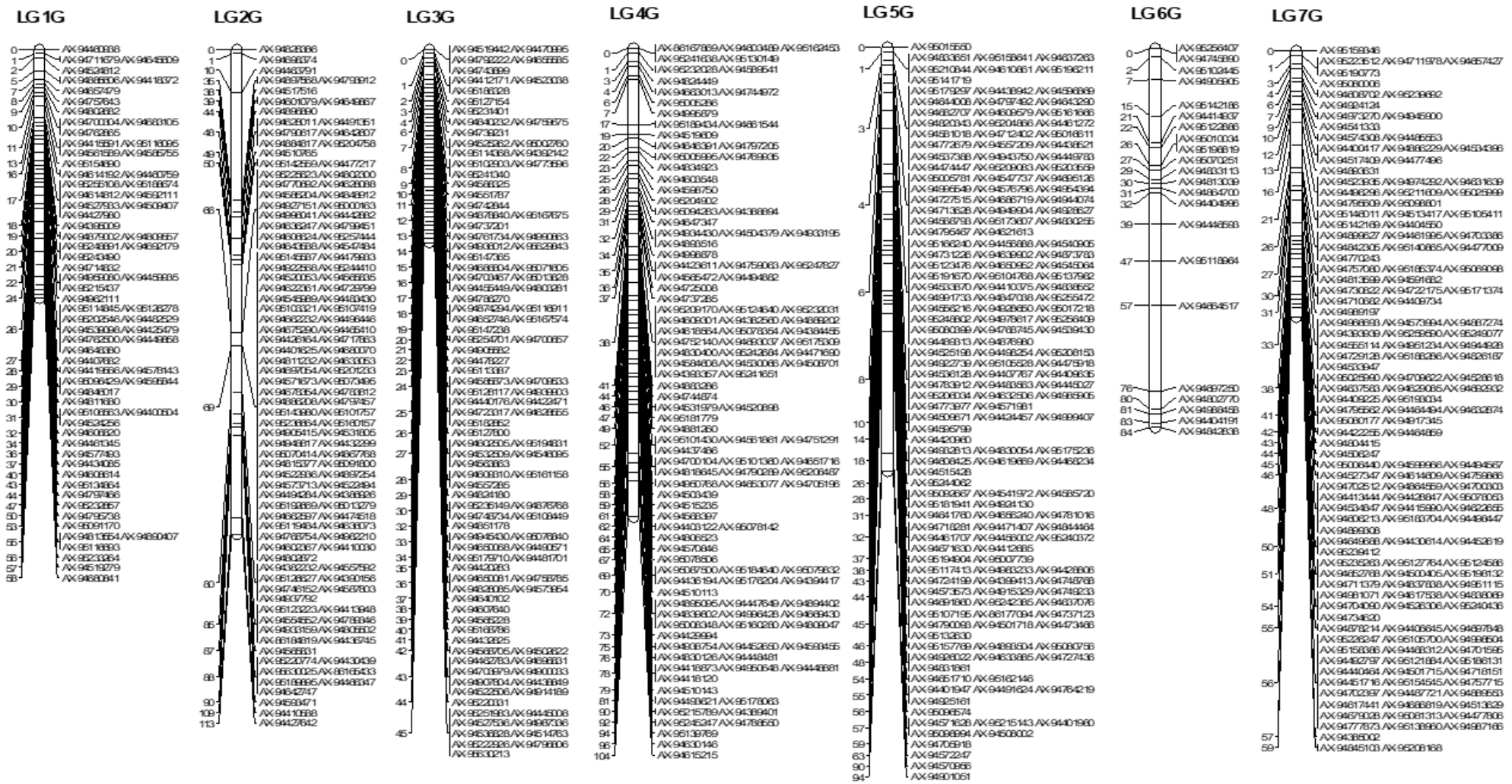

Figure 10 - Genetic linkage map of the G genome of *T. timopheevii*. SNP marker names and cM distances for each of the seven linkage groups are also shown in appendix A.

4.4 Preferential transmission

The data obtained from SNP analysis was used to determine the transmission frequency of chromosome segments (Table 5) from each of the fourteen *T. timopheevii* linkage groups to the female gametes of the BC_1, BC_2, BC_3 (F_1), BC_4 (BC_2) generations. The number of introgressions was mostly higher for the A^t genome than the G genome, excepted for 2G and 6G. The Linkage groups 2G and $5A^t$ showed the highest rates of transmission and this pattern was maintained from BC_1 to BC_4 generations. Chromosome segments from linkage group $5A^t$ were observed in 100% of the BC_1 plants but it decreased over the generations reaching 49% at the BC_4 generation. The same happened for the 2G linkage group, that was presented on 93% of the BC_1 and reached 57% at the BC_4 generation.

Table 5 - Transmission frequencies of the fourteen linkage groups of *T. timopheevii* in the backcross populations analysed by SNP genotyping

	BC_1		BC_2		BC_3 (F_1)		BC_4 (BC_1)		Total	
Number of plants genotyped	14	(%)	27	(%)	60	(%)	35	(%)	136	(%)
Linkage group $1A^t$	10	71	13	48	15	25	3	9	41	30
Linkage group 1G	5	36	5	19	7	12	1	3	18	13
Linkage group $2A^t$	9	64	6	22	5	8	1	3	21	15
Linkage group 2G	13	93	23	85	38	63	20	57	94	69
Linkage group $3A^t$	7	50	11	41	15	25	5	14	38	29
Linkage group 3G	9	64	4	15	8	13	2	6	23	17
Linkage group $4A^t$	12	86	14	52	16	27	3	9	45	33
Linkage group 4G	11	79	9	33	13	22	5	14	38	28
Linkage group $5A^t$	14	100	23	85	38	63	17	49	92	68
Linkage group 5G	11	79	12	44	17	28	9	26	49	36
Linkage group $6A^t$	13	93	15	56	17	28	5	14	50	37
Linkage group 6G	10	71	15	56	19	32	14	40	58	43
Linkage group $7A^t$	10	71	10	37	14	23	2	6	36	26
Linkage group 7G	10	71	7	26	8	13	3	9	28	21

In general, the number of putative introgressions/segments decreased over the generations, which was expected since a backcross approach was used to recover most of the genotype from the recurrent parent, and to reduce the genotype from the donor. However, the number of backcrosses needed to recover the recurrent parent is unpredictable, the heritability of the desired characters has to be considered but also the linkage drag which may increase the number of backcrosses. Many factors influence the preferential transmission of segments in interspecific hybrids including, the homology between the related species.

The degree of divergence between the A^t an A genomes is less than that between the G and B genomes (HUANG et al., 2002; RODRÍGUEZ et al., 2000; SALINA et al., 2006). That explains why most of the introgressions come from linkage group A^t as they have higher level of recombination with the A genome of wheat. However, an exception is observed for linkage group 2G, which is the most frequent after four backcrosses. Brown-guedira et al. (1996) analysed hybrids between a *T. timopheevii-Ae. tauschii* amphiploid and CS nulisomic-tetrasomic lines and they found that after one to four backcrosses to wheat, chromosome 2G was detected in 68% of the lines, our data shows similar frequency, after four backcrosses linkage group 2G was detected in 57% of the lines. The chromosome 2G has a gametocidal gene resulting in its preferential transmission (NYQUIST, 1962; TIMONOVA et al., 2013),

Badaeva et al. (1991) compared hybrids between different combination of *T. aestivum* cultivars with *T. timopheevii* and they found that the pattern of chromosome substitutions and rearrangements were unequal and depended on the genotype of *T. aestivum* parental cultivar, so they confirmed the influence of wheat background on the introgression process (SHKUTINA; KALININA; USOVA, 1988).

The SNP analysis does not reveal which wheat chromosome has been involved in introgressions with *T. timopheevii,* thus the work described here does not allow to detect in which wheat genome the introgressions are happening. The Wheat Research Centre at UoN is developing KASP markers with the aim of rectifying this problem. KASP marker will be wheat chromosome specific allowing to detect in which wheat chromosome the recombination event is happening.

4.5 Syntenic relationship between wheat and *T. timopheevii*

A total of 1,432 markers based on the genetic map of *T. timopheevii* were used in BLAST against wheat Chinese Spring genome assembly to obtain the orthologous map positions of the top hits in the A, B and D genomes of wheat.

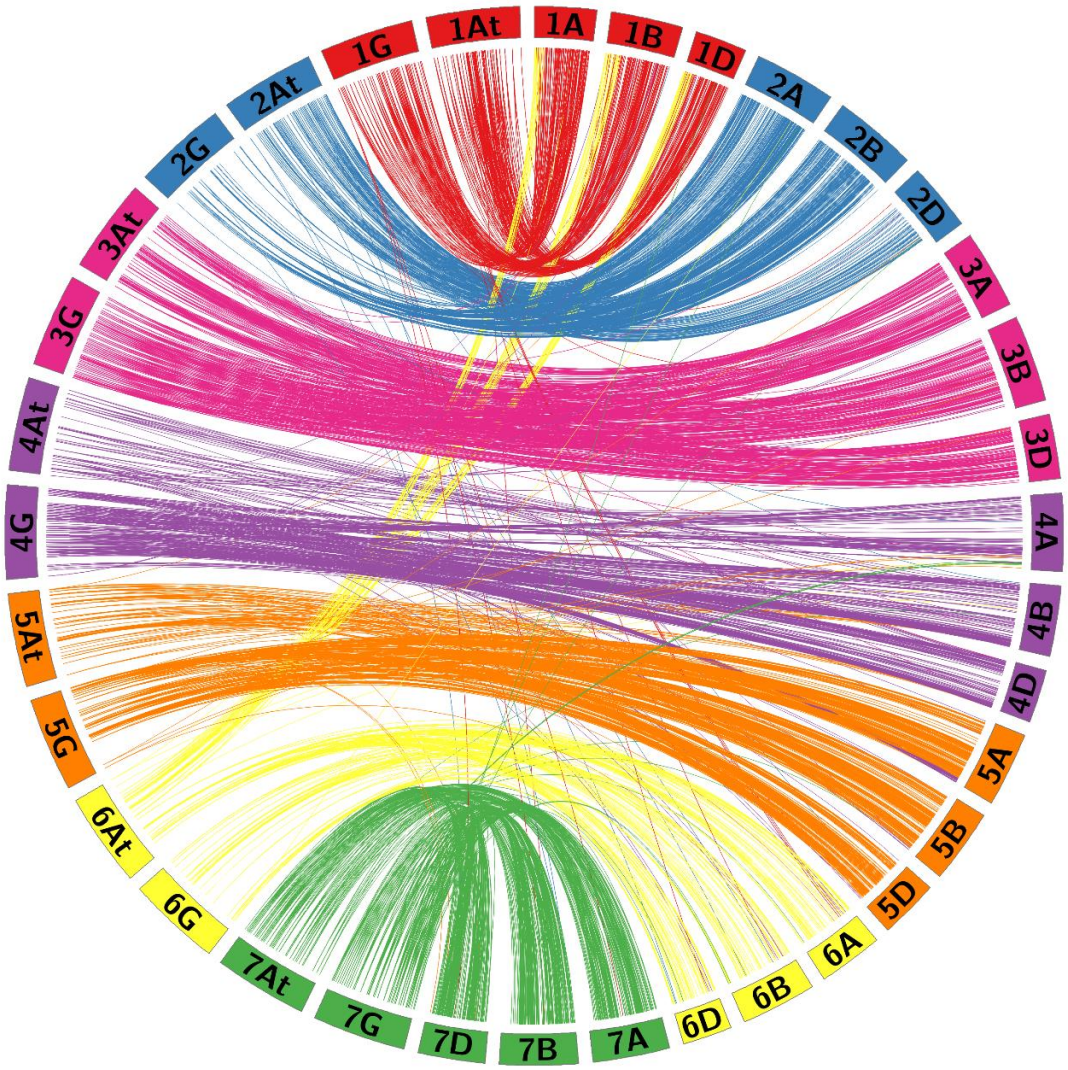

Figure 11 - Synteny of *T. timopheevii* (genetic position in cM) with hexaploid wheat (physical position in Mb).

Figure 11 shows the syntenic relationships between the seven linkage groups of each of the two genomes of *T. timopheevi* and the seven linkage groups of each of the three genomes of wheat with large 'ribbons' showing significant synteny. Some gene rearrangements are indicated in the diagram where usually single markers cross map to non-collinear positions on the wheat chromosomes.

SNP analysis showed a close syntenic relationship between the three genomes of wheat and the A^t and G genomes of *T. timopheevii.* Syntenic analysis performed here was able to show the disruption of synteny between *T. aestivum* and *T. timopheevii,* since the species-specific 4A-5A-7B cyclic translocation presented in durum (*T. turgidum*) and common wheat (*T. aestivum*) (LIU et al., 1992; NARANJO et al., 1987) is lacking in *T. timophevii*.

According to the diphyletic origin *T. timopheevii* and *T. turgidum (genome AABB,* species involved in the *T. aestivum* origin*)* arose from two different hibridisation events. The presence of the 4A-5A-7B translocation in durum and commom wheat and its absence in *T. timopheevii* is one evidence that supports this hypothesis, and the markers used for the syntenic analysis in this study were able show it, since no markers from 7B were mapped on chromosome $4A^t$ of *T. timopheevii*. However, no significant markers from 7G of *T. timopheevvi* were mapped to 4A of wheat, the green line crossing 4A/7G at the Circos plot.

As stated before, it is suggested that an ancestral form of *T. urartu* was the donor of A and A^t genome, and the divergence between them is about 0.5 MYA. *T. urartu* presents a 4AL/5AL translocation (KING et al., 1994), which was transmitted to *T. turgidum* and *T. aestivum* (NARANJO et al., 1987) and it was also reported in *T. timopheevii*. A conserved order of markers in the 4AL/5AL translocation region was found in $4A^tL/5A^tL$.

The donors of A, B/G, and D genomes of the polyploid wheats diverged about 2.5–4.5 MYA (HUANG et al., 2002; LEVY, 2002). The tetraploid wheats *T. dicoccoides* (AABB) and *T. timopheevii* (A^tA^tGG) appeared about 0.5 MYA. *T. timpophevii* carry a $3A^tL/4A^tL$ translocation and which is not present in *T. turgidum* (MAESTRA; NARANJO, 1999; RODRÍGUEZ et al., 2000). Our genetic mapping and comparative analysis showed a few markers from 3A mapped to $4A^t$. We also found on chromosome $4A^t$, markers from 5A, 3A and 6A of wheat, which are reported to be

part of $4A^t$ chromosome after a sequence of translocations (MAESTRA; NARANJO, 1999; RODRÍGUEZ et al., 2000).

Syntenic analysis performed here was able to show the $6A^tS/1GS$ cyclic translocation reported before (GILL; CHEN, 1987; MAESTRA; NARANJO, 1999; RODRÍGUEZ et al., 2000). A significant number of markers from short arm of chromosome 1G where mapped onto group 6 of wheat, and markers from chromosome $6A^t$ where mapped onto group 1 of wheat (Figure 11). The mc-GISH analysis in Figure 6b shows a A^t/G translocation and thus, this result confirms that the translocation is actually $6A^tS/1GS$ as previously reported.

Thus, this analysis represents a close syntenic relationship between *T. timopheevii* and the A, B and D genomes of wheat, it also shows a series of species-specific translocations that happen over the years and according to Maestra and Naranjo (1999), were most likely produced at early tetraploid stages, i.e., after the split from *T. urartu*, as a way to genome organisation immediately after polyploidy formation.

5. Conclusions

From the data obtained here *T. timopheevii* shows a good potential to be used in introgression programs because its introgression lines present high germination rates, fertility level and good seed production, compared to other wild species normally used in such programs.

The number of individuals and frequency of recombination enabled the construction of a genetic map of *T. timopheevii*, with a large number of markers well distributed along the whole the chromosome, and that could be used to identify *T. timopheevii* introgression in a wheat background.

The markers used in the comparative analysis showed a close syntenic relationship between *Triticum timopheevii* and *Triticum aestivum* .

References

ABLE, J. A.; LANGRIDGE, P.; MILLIGAN, A. S. Capturing diversity in the cereals: many options but little promiscuity. **Trends in Plant Science**, v. 12, n. 2, p. 71-79, 2007.

ABROUK, M. et al. The *in silico* identification and characterization of a bread wheat/*Triticum militinae* introgression line. **Plant Biotechnology Journal**, v. 15, n. 2, p. 249-256, 2017.

AKBARI, M. et al. Diversity arrays technology (DArT) for high-throughput profiling of the hexaploid wheat genome. **Theoretical and Applied Genetics**, v. 113, n. 8, p. 1409-1420, 2006.

AL-KAFF, N. et al. Detailed dissection of the chromosomal region containing the Ph1 locus in wheat *Triticum aestivum*: with deletion mutants and expression profiling. **Annals of Botany**, v. 101, n. 6, p. 863-872, 2008.

ALLABY, R. G.; BROWN, T. A. Identification of a 5S rDNA spacer type specific Triticum urartu and wheats containing the *T. urartu* genome. **Genome**, v. 43, p. 250-254, 2000.

BADAEVA, E. D. et al. General features of chromosome substitutions in *Triticum aestivum* x *T. timopheevii* hybrids. **Theoretical and Applied Genetics**, p. 227-232, 1991.

BARCELLOS, A. L.; MORAES-FERNANDES, M. I. B. DE; ROELFS, A. P. Ferrugem da folha do trigo (*Puccinia recondita*): durabilidade da resistência. **Summa Phytopathologica**, v. 23, n. 2, p. 101-117, 1997.

BORÉM, A.; SCHEEREN, P. L. **Trigo: do plantio à colheita.** Viçosa: UFV, 2017.

BREIMAN, A.; GRAUR, D. Wheat evolution. **Israel Journal of Plant Sciences**, v. 43:2, p. 85-98, 1995.

BROWN-GUEDIRA, G. L. et al. Chromosome substitutions of *Triticum timopheevii* in common wheat and some observations on the evolution of polyploid wheat species. **Theoretical and Applied Genetics**, n. 93, p. 1291-1298, v. 93, p. 1291-1298, 1996.

BROWN-GUEDIRA, G. L.; SINGH, S.; FRITZ, A. K. Performance and Mapping of Leaf Rust Resistance Transferred to Wheat from *Triticum timopheevii* subsp. *armeniacum*. **Phytopathology**, v. 93, n. 7, p. 784-789, 2003.

CASA, R. T.; REIS, E. M. **No TitleDoenças de cereais de inverno: guia de campo para identificação e controle**. Lages: Graphel, 2014.

CASSMAN, K. G. Ecological intensification of cereal production systems: yield potential, soil quality, and precision agriculture. **Proceedings of the National Academy of Sciences of the United States of America**, v. 96, n. 11, p. 5952-9, 1999.

CAVANAGH, C. R. et al. Genome-wide comparative diversity uncovers multiple targets of selection for improvement in hexaploid wheat landraces and cultivars. **Proceedings of the National Academy of Sciences**, v. 110, n. 20, p. 8057-8062, 2013.

CHAUDHARY, H. K.; KAILA, V.; RATHER, S. A. Wheat. In: PRATAP, A.; KUMAR, J. (Eds.). **Alien Gene Transfer in Crop Plants, Volume 2: Achievements and Impacts**. Palampur: Springer, 2014.

CONAB - Companhia Nacional de Abastecimento. **Acompanhamento da Safra Brasileira - Grãos. v.5, Safra 2017/18 -** Décimo levantamento, Brasília: 2018.

COX, T. S. Deepening the Wheat Gene Pool Deepening the Wheat Gene Pool. **Journal of Crop Production**, v. 1, n. 1, p. 1-25, 1998.

CURTIS, B.; RAJARAM, S.; MACPHERSON, H. **Bread wheat: improvement and production.** 30. ed. Rome: FAO, 2002.

DEVI, U. **The development of introgression lines of *Triticum timopheevii* in a cultivated wheat background and their characterisation**. Thesis. University of Nottingham, 2017.

DOVER, G. A.; RILEY, R. Variation at Two Loci affecting Homoeologous Meiotic Chromosome Pairing in *Triticum aestivum* × *Aegilops mutica* Hybrids. **Nature New Biology**, v. 235, n. 54, p. 61-62, 1972.

DUBCOVSKY, J.; DVORAK, J. Genome Plasticity a Key Factor. **Science**, v. 1862, v. 316, 2007.

DVORAK, J. et al. The evolution of polyploid wheats: identification of the A genome donor species. **Genome**, v. 36, n. 1, p. 21-31, 1993.

DVORAK, J. *Triticum* Species (Wheat). **Encyclopedia od Genetics**, p. 2060-2068, 2001.

DVORAK, J.; ZHANG, H. B. Variation in repeated nucleotide sequences sheds light on the phylogeny of the wheat B and G genomes. **Proceedings of the National Academy of Sciences of the United States of America**, v. 87, n. 24, p. 9640-9644, 1990.

DYCK, P. L. Transfer of a gene for stem rust resistance from *Triticum araraticum* to hexaploid wheat. **Genome**, v. 35, n. 5, p. 788-792, out. 1992.

DYCK, P. L.; SAMBORSKI, D. J. **Host-parasite interactions involving two genes for leaf rust resistance in wheat.** In: F. W. Findlay, K. W. Sheperd, (Eds.) 3rd International Wheat Genetics Symposium. **Anais**. Canberra: Australian Academy of Science, 1968

ENDO, T. R. Gametocidal chromosomes and their induction of chromosome mutations in wheat. **The Japanese Journal of Genetics**, v. 65, n. 3, p. 135-152, 1990.

EILAM, T. et al. Genome size and genome evolution in diploid Triticeae species. **Genome**, v. 50, n. 11, p. 1029-1037, 2007.

FAO - Food and Agrigulture Organization of the United Nations. **Statistical Yearbook 2005-2006**. Rome: 2006.

FAOSTAT - Statistical databases. **Annual Crop Production Statistics.** Rome, 2018. Available at: <http://.fao.org/faostat/en/#data/TP> Acessed june 2018.

FAO - Food and Agrigulture Organization of the United Nations **The future of food and agriculture: Trends and challenges,** Rome: 2018

FELDMAN, M. Origin of cultivated wheat. In: BONJEAN, A.; ANGUS, W. (Eds.). **The World Wheat Book: A History of Wheat Breeding**. Lavoisier ed. Paris: p. 3-56.

FELDMAN, M.; LEVY, A. A. Allopolyploidy - A shaping force in the evolution of wheat genomes. **Cytogenetic and Genome Research**, v. 109, n. 1-3, p. 250-258, 2005.

FEUILLET, C.; LANGRIDGE, P.; WAUGH, R. Cereal breeding takes a walk on the wild side. **Trends Genet**, v. 24, n. 1, p. 24-32, 2007.

FRIEBE, B. et al. Characterization of wheat-alien translocations conferring resistance to diseases and pests: Current status. **Euphytica**, n. 91, n. 1, p. 59-87, 1996.

FRIEBE, B.; GILL, B. S. Chromosome banding and genome analysis in diploid and cultivated polyploid wheats. In: P. P. Jauhar (Ed.). **Methods of Gneome Analysis in Plants**. Florida: CRC Press, 1996. p. 39-59.

GALL, J. G.; PARDUE, M. L. Formation and detection of RNA-DNA hybrid molecules in cytological preparations. **Proceedings of the National Academy of Sciences**, 1969.

GILL, B. S.; CHEN, P. D. Role of cytoplasm-specific introgression in the evolution of the polyploid wheats. **Proceedings of the National Academy of Sciences of the United States of America**, v. 84, n. 19, p. 6800-6804, 1987.

GILL, B. S.; RAUPP, W. J. Direct Genetic Transfers from *Aegilops squarrosa* L. to Hexaploid Wheat. **Crop Science**, 1987.

GILL, B. S.; SEARS, R. G. **T**he Current Status of Chromosome Analysis in Wheat. In: J.P Gustafson and R. Apples (Eds.) C**hromosome structure and function.** Pleno publishing Corp, New York**,** 1988.

GREWAL, S. et al. Characterisation of *Thinopyrum bessarabicum* chromosomes through genome-wide introgressions into wheat. **Theoretical and Applied Genetics**, v. 131, n. 2, p. 389-406, 2018.

GRIFFITHS, S. et al. Molecular characterization of *Ph1* as a major chromosome pairing locus in polyploid wheat. **Nature**, v. 439, n. 7077, p. 749–752, 2006.

GUINNESS. **Guinness world records 2017**. LONDON: Guinness World Record Limited, 2017.

GUSTAFSON, J. P.; DERA, A. R. Alien gene manipulation and expression in wheat1. **Genome**, v. 31, 1989.

GYARFAS, J. **Transference of disease resistance from *Triticum timopheevii* to *Triticum aestivum*.** Thesis. University of Sydney, Australia, 1978.

HALDANE, J. B. S. The probable errors of calculated linkage values, and the most accurate method of determining gametic from certain zygotic series. **Journal of Genetics**, v. 8, n. 4, p. 291-297, 1919.

HANSON, H.; BORLAUG, N. E.; ANDERSON, R. G. **Wheat in the Third World.** Boulder: lADS Development Oriental Literature Series. Westview Press., 1982.

HARLAN, J. R.; WET, J. M. J. Toward a Rational Classification of Cultivated Plants. **Plant Taxonomy**, v. 20, n. 4, p. 509-517, 1971.

HAUDRY, A. et al. Grinding up Wheat: A Massive Loss of Nucleotide Diversity Since Domestication. **Mol. Biol. Evol**. v.24, n.7, p. 1506-1571, 2007.

HELMSJØRGENSEN, J.; JENSEN, C. J. Gene *Pm6* for resistance to powdery mildew in wheat. **Euphytica**, v. 22, n. 2, p. 423–423, jun. 1973.

HUANG, S. et al. Genes encoding plastid acetyl-CoA carboxylase and 3-phosphoglycerate kinase of the *Triticum/Aegilops* complex and the evolutionary history of polyploid wheat. **Proc. Natl Acad. Sci. USA**, v. 99, p. 8133–8138, 2002.

IWGSC. A chromosome-based draft sequence of the hexaploid bread wheat (*Triticum aestivum*) genome. **Science**, v. 345, n. 6194, 2014.

JÄRVE, K. et al. Chromosomal location of a *Triticum timopheevii*-derived powdery mildew resistance gene transferred to common wheat. **Genome**, v. 43, n. 2, p. 377-381, 2000.

JARVE, K.; JAKOBSON, I. Tetraploid wheat species *Triticum timopheevii* and *Triticum militinae* in common wheat improvement. **Acta Agronomica**, v.50, n.4, p. 463-477, 2002.

JONES, G. H. The control of chiasma distribition. **Symp Soc Exp Biol.**, v. 38, p. 293–320, 1984.

JONES, G. H.; FRANKLIN, F. C. H. Meiotic, Crossing-over: Obligation and Interference. **Cell**, v. 126, n. 2, p. 246–248, 2006.

KATO, A.; LAMB, J. C.; BIRCHLER, J. A. Chromosome painting using repetitive DNA sequences as probes for somatic chromosome identification in maize. **Proceedings of the National Academy of Sciences**, 2004.

KIHARA, H. Ueber cytologische Studien bei einigen Getreidearten. Spezies-Bastarde des Weizen und Weizenroggen-Bastard. **Bot Mag**, v. 33, p. 17–38, 1919.

KIHARA, H. Cytologische und genetische Studien bei wichtigen Getreidearten mit besonderer Riicksicht auf das Verhalten der Chromosomen und die Sterilitat in den Bastarden. **Mem. Coll, Sci. Kyoto Imp Univ.**, v. 1B, p. 1-200, 1924.

KIHARA, H. Discovery of the DD-analyser, one of the ancestors of *Triticum vulgare* (abstr) (in Japanese). **Agric. Hortic.**, v. 19, p. 889-890, 1944.

KIHARA, H. Considerations on the Evolution and Distribution of *Aegilops* Species Based on the Analyser-method. **Cytologia**, v. 19, n. 4, p. 336-357, 1954.

KIHARA, H.; YAMASHITA, K.; TANAKA, M. Genomes of 6x species of *Aegilops*. **Wheat. Inf. Serv.**, v. 8, p. 3-5, 1959.

KILIAN, B. et al. Independent wheat B and G genome origins in outcrossing Aegilops progenitor haplotypes. **Molecular Biology and Evolution**, v. 24, n. 1, p. 217-227, 2007a.

KILIAN, B. et al. Molecular diversity at 18 loci in 321 wild and 92 domesticate lines reveal no reduction of nucleotide diversity during *Triticum monococcum* (einkorn) domestication: Implications for the origin of agriculture. **Molecular Biology and Evolution**, v. 24, n. 12, p. 2657-2668, 2007b.

KING, I. P. et al. Detection of Interchromosomal Translocations Within the Triticeae By Rflp Analysis. **Genome**, v. 37, n. 5, p. 882-887, 1994.

KING, J. et al. A step change in the transfer of interspecific variation into wheat from *Amblyopyrum muticum*. **Plant Biotechnology Journal**, v. 15, n. 2, p. 217-226, 2017.

KING, J. et al. Introgression of *Aegilops speltoides* segments in *Triticum aestivum* and the effect of the gametocidal genes. **Ann. Bot.**, v. 121, n. 1, p. 229-240, 2018.

KOO, D. H. et al. Homoeologous recombination in the presence of *Ph1* gene in wheat. **Chromosoma**, v. 126, n. 4, p. 531-540, 2017.

KRZYWINSKI, M. ET AL. Circos: an Information Aesthetic for Comparative Genomics. **Genome Res**, v. 19, n. 604, p. 1639-1645, 2009.

LEONOVA, I. et al. Identification of microsatellite markers for a leaf rust resistance gene introgressed into common wheat from *Triticum timopheevii*. **Plant Breeding**, v. 123, n. 1, p. 93-95, 2008.

LEONOVA, I. N. et al. Microsatellite Mapping of a Leaf Rust Resistance Gene Transferred to Common Wheat from *Triticum timopheevii*. **Cereal Research Communications**, v. 38, n. 2, p. 211-219, 2010.

LEONOVA, I. N. et al. *Triticum aestivum* − *Triticum timopheevii* Introgression Lines as a Source of Pathogen Resistance Genes. **Czech J. Genet. Plant Breed.,** v. 47 p. 49–55, 2011.

LEVY, A. A. The Impact of Polyploidy on Grass Genome Evolution. **Plant Physiology**, v. 130, n. 4, p. 1587–1593, 2002.

LIU, C. J. et al. Non-homoeologous translocations between group 4, 5 and 7 chromosomes within wheat and rye. **Theoretical and Applied Genetics**, v. 83, n. 3, p. 305-312, 1992.

MA, H.; HUGHES, G. R. Genetic control and chromosomal location of *Triticum timopheevii*-derived resistance to septoria nodorum blotch in durum wheat. **Genome**, v. 38, n. 2, p. 332-338, 1995.

MAESTRA, B.; NARANJO, T. Structural chromosome differentiation between *Triticum timopheevii* and *T. turgidum* and *T. aestivum*. **Theoretical and Applied Genetics**, v. 98, n. 5, p. 744–750, 1999.

MARTÍN, A. C. et al. Dual effect of the wheat *Ph1* locus on chromosome synapsis and crossover. **Chromosoma**, v. 126, n. 6, p. 669-680, 2017.

MASOUDI-NEJAD, A. et al. Transfer of rye chromosome segments to wheat by a gametocidal system. **Chromosome research**, v. 10, n. 5, p. 349-57, 2002.

MATSUOKA, Y. Evolution of Polyploid Triticum Wheats under Cultivation: the role of domestication, natural hybridization and allopolyploid speciation in their diversification. **Plant Cell Physiol**, v. 52, n. 5, p. 750-764, 2011.

MAXWELL, J. J. et al. MlAG12: a *Triticum timopheevii* -derived powdery mildew resistance gene in common wheat on chromosome 7AL. **Theor. Appl. Gent.**, v.119, p. 1489-1495, 2009.

MCFADDEN, E. S.; SEARS, E. R. The artificial synthesis of *Triticum spelta*. **Rec. Genet. Soc. Am.**, v. 13, p. 26-27, 1944.

MCFADDEN, E. S.; SEARS, E. R. The origin of *Triticum spelta* and its free-threshing hexaploid relatives. **Journal of Heredity**, v. 37, n. 3, p. 81-89, 1946.

MCINTOSH, R. A. **Genetic and cytogenetic studies involving *Lr18* for resistance to *Puccinia recondita*.** (S. Sakamota, Ed.) Proceedings of the 6th International Wheat Genetics Symposium Kyoto. **Anais**. Yokohama: 1983

MCINTOSH, R. A. et al. **Catalogue of gene symbols for wheat**. (Y. Ogihara, Ed.) Proceedings of the 12th international wheat genetics symposium. **Anais**.Yokohama,: 2013

MCINTOSH, R. A.; GYARFAS, J. *Triticum timopheevi* as a source of resistance to wheat stem rust. **Zeitschrift fur Pflanzenzuchtung**, v. 66, p. 240-248, 1971.

MIKÓ, P. et al. Molecular cytogenetic identification and phenotypic description of a new synthetic amphiploid, *Triticum timococcum* ($A^tA^tGGA^mA^m$). **Genetic Resources and Crop Evolution**, v. 62, p. 55-66, 2014.

MILNE, I. et al. Flapjack-graphical genotype visualization. **Bioinformatics**, v. 26, n. 24, p. 3133–3134, 2010.

MIYASHITA, N. T.; MORI, N.; TSUNEWAKI, K. Molecular variation in chloroplast DNA regions in ancestral species of wheat naohiko. **Genetics Society of America Molecular**, v. 137, p. 883-889, 1994.

NARANJO, T. et al. Arm homoeology of wheat and rye chromosomes. **Genome**, 2v. 20, n. 6, p. 873-882, 1987.

NIRANJANA, M. Gametocidal genes of *Aegilops*: segregation distorters in wheat–*Aegilops* wide hybridization. **Genome**, v. 60, n. 8, p. 639-647, 2017.

NYQUIST, W. Differential fertilization in the inheritance of stem rust resistance in hybrids involving a common wheat strain derived from *Triticum timopheevi*. **Genetics**, v. 47, p. 1109-1124, 1962.

OECD/FAO. **OECD-FAO Agricultural Outlook 2012-2021**. OECD Publishing and FAO, 2012.

OETTLER, G. **T**he fortune of a botanical curiosity - *Triticale*: Past, present and future. **Journal of Agricultural Science**, v. 143, n. 5, p. 329-346, 2005.

OGIHARA, Y.; TSUNEWAKI, K. Diversity and evolution of chloroplast DNA in *Triticum* and *Aegilops* as revealed by restriction fragment analysis. **Theoretical and Applied Genetics**, v. 76, n. 3, p. 321-332, 1988.

PERUGINI, L. D. et al. *Pm37*, a new broadly effective powdery mildew resistance gene from *Triticum timopheevii*. **Theoretical and Applied Genetics**, v. 116, n. 3, p. 417-425, 2008.

POLAND, J. A. et al. Development of high-density genetic maps for barley and wheat using a novel two-enzyme genotyping-by-sequencing approach. **PLoS ONE**, v. 7, n. 2, 2012.

RAY, D. K. et al. Yield trends are insufficient to double global crop production by 2050. **PLoS ONE**, v. 8, n. 6, 2013.

REIF, J. C. et al. Wheat genetic diversity trends during domestication and breeding. **Theoretical and Applied Genetics**, v. 110, n. 5, p. 859-864, 2005.

REY, M. D. et al. Exploiting the *ZIP4* homologue within the wheat *Ph1* locus has identified two lines exhibiting homoeologous crossover in wheat-wild relative hybrids. **Molecular Breeding**, v. 37, n. 8, p. 95, 2017.

REYNOLDS, M. et al. Achieving yield gains in wheat. **Plant, Cell and Environment**, v. 35, n. 10, p. 1799-1823, 2012.

RILEY, R.; CHAPMAN, V. Genetic control of the cytologically diploid behaviour of hexaploid wheat. **Nature Plants**, v. 182, p. 713-715, 1958.

ROBERTS, M. A. et al. Induction and characterization of *Ph1* wheat mutants. **Genetics**, v. 153, n. 4, p. 1909-1918, 1999.

RODRÍGUEZ, S. et al. Chromosome structure of *Triticum timopheevii* relative to *T. turgidum*. **Genome**, v. 43, n. 6, p. 923-930, 2000.

ROUSSEL, V. et al. Molecular diversity in French bread wheat accessions related to temporal trends and breeding programmes. **TAG Theoretical and Applied Genetics**, v. 108, n. 5, p. 920-930, 2004.

SAINTENAC, C. et al. Sequence-based mapping of the polyploid wheat genome. **G3: Genes|Genomes|Genetics**, v. 3, n. 7, p. 1105-1114, 2013.

SAKAMURA, T. Kurze Mitteilung über die Chromosomenzahlen und die Verwandtschaftsverhältnisse der Triticum-Arten. **Bot Mag Tokyo**, v. 32, p. 151-151, 1918.

SALAMINI, F. et al. Genetics and geography of wild cereal domestication in the near east**. Nature Reviews Genetics**, v. 3, p. 429-441, 2002.

SALINA, E. A. et al. Wheat genome structure: Translocations during the course of polyploidization. **Functional and Integrative Genomics**, v. 6, n. 1, p. 71-80, 2006.

SASANUMA, T.; MIYASHITA, N. T.; TSUNEWAKI, K. Wheat phylogeny determined by RFLP analysis of nuclear DNA. 3. Intra-and interspecific variations of five *Aegilops* Sitopsis species. **Theoretical and Applied Genetics**, v. 92, n. 8, p. 928-934, 1996.

SAX, K. Sterility in wheat hybrids. II. Chromosome behavior in partially sterile hybrids. **Maine Agricultural Experiment Station**, v. 7, n. 6, p. 513-551, 1922.

SCHNEIDER, A.; MOLNÁR, I.; MOLNÁR-LÁNG, M. Utilisation of *Aegilops* (goatgrass) species to widen the genetic diversity of cultivated wheat**. Euphytica**, v. 163, n. 1, p. 1-19, 2008.

SCHWARZACHER, T. et al. Genomic in situ hybridization to identify alien chromosomes and chromosome segments in wheat. **Theoretical and Applied Genetics**, v. 84, n. 7-8, p. 778-786, 1992.

SEARS, E. R. Wheat Cytogenetics. **Annual Review of Genetics**, v. 3, n. 1, p. 451-468, 1969.

SEARS, E. R. An induced mutant with homoeologous pairing in common wheat. **Can. J. Genet. Cytol**, v. 19, p. 585–593, 1977.

SEARS, E. R. Transfer of alien genetic material to wheat. **Transfer of alien genetic material to wheat.**, p. 75–89, 1981.

SEARS, E. R.; GUSTAFSON, J. P. Use of Radiation to Transfer Alien Chromosome Segments to Wheat. **Crop Science**, v. 33, n. 5, p. 897, 1993.

SHKUTINA, F. M.; KALININA, N. P.; USOVA, T. K. The role of *Triticum aestivum* L. variations in the level of introgression of allogenic genetic material into its genome and in the rate of stabilization of the hybrid form. **Genetika**, v. 24, p. 98-109, 1988.

TAKUMI, S. et al. Wheat phylogeny determined by RFLP analysis of nuclear DNA. 1. Einkorn wheat. **Japanese Journal of Genetics**, v. 68, p. 73-79, 1993.

TANKSLEY, S. D.; MCCOUCH, S. R. Seed Banks and Molecular Maps: Unlocking Potential from the Wild Genetic Base of Crop Plants. **Science**, v. 277, n. 5329, p. 1063-1066, 1997.

TANNO, K.; WILCOX, G. How Fast was wild wheat domesticated? **Science**, v. 311, p. 1886, 2006.

TILMAN, D. et al. Global food demand and the sustainable intensification of agriculture. **Proceedings of the National Academy of Sciences**, v. 108, n. 50, p. 20260-20264, 2011.

TIMONOVA, E. M. et al. Marker-assisted development and characterization of a set of *Triticum aestivum* lines carrying different introgressions from the *T. timopheevii* genome. **Molecular Breeding**, v. 31, n. 1, p. 123-136, 2013.

TIWARI, V. K. et al. SNP Discovery for mapping alien introgressions in wheat. **BMC Genomics**, v. 15, n. 1, p. 1-11, 2014.

TIWARI, V. K. et al. Exploring the tertiary gene pool of bread wheat: Sequence assembly and analysis of chromosome 5Mgof *Aegilops geniculata*. **Plant Journal**, v. 84, n. 4, p. 733-746, 2015.

VAN BERLOO, R. GGT 2.0: Versatile software for visualization and analysis of genetic data. **Journal of Heredity**, v. 99, n. 2, p. 232-236, 2008.

VAN OOIJEN, J. W. Multipoint maximum likelihood mapping in a full-sib family of an outbreeding species. **Genetics Research**, v. 93, n. 5, p. 343-349, 2011.

VENSKE, E. **Estratégias de avanço no melhoramento genético de trigo: enriquecimento do germoplasma brasileiro com introgressões de Aegilops speltoides e o QTLoma da resistência à giberela**. Tese, Universidade Federal de Pelotas, 2017.

VOORRIPS, R. E. MapChart: Software for the Graphical Presentation of Linkage Maps and QTLs. **Journal of Heredity**, v. 93, n. 1, p. 77-78, 2002.

WAGENAAR, E. B. Studies on the genome constitution of *Triticum timopheevii* zhuk. I. Evidence for genetic control of meiotic irregularities in tetraploid hybrids. **Can. J. Genet. Cytol.** n. 46, p. 47-60, 1961.

WANG, D. Wide hybridization: engineering the next leap in wheat yield. **Journal of Genetics and Genomics**, v. 36, n. 9, p. 509-510, 2009.

WANG, S. et al. Characterization of polyploid wheat genomic diversity using a high-density 90 000 single nucleotide polymorphism array. **Plant Biotechnology Journal**, v. 12, n. 6, p. 787-796, ago. 2014.

WHITE, J. et al. The genetic diversity of UK, US and Australian cultivars of *Triticum aestivum* measured by DArT markers and considered by genome. **Theoretical and Applied Genetics**, v. 116, n. 3, p. 439-453, 2008.

WICKER, T. et al. Frequent gene movement and pseudogene evolution is common to the large and complex genomes of wheat, barley, and their relatives. **The Plant Cell**, v. 23, n. 5, p. 1706-1718, 2011.

WILKINSON, P. A. et al. CerealsDB 2.0: an integrated resource for plant breeders and scientists. **BMC Bioinformatics**, v. 13, n. 219, 2012.

WILKINSON, P. A. et al. CerealsDB 3.0: Expansion of resources and data integration. **BMC Bioinformatics**, v. 17, n. 1, p. 1-9, 2016.

WINFIELD, M. O. et al. High-density SNP genotyping array for hexaploid wheat and its secondary and tertiary gene pool. **Plant. Biotechnol. J**., v. 796, p. 1195-1206, 2016.

WU, S.; PUMPHREY, M.; BAI, G. Molecular mapping of stem-rust-resistance gene *Sr40* in wheat. **Crop Science**, 2009.

YAMAMORI, M. An N-band marker for gene *Lr18* for resistance to leaf rust in wheat. **Theoretical and Applied Genetics**, 1994.

ZHANG, H. et al. Persistent whole-chromosome aneuploidy is generally associated with nascent allohexaploid wheat. **Proceedings of the National Academy of Sciences**, 2013.

ZHANG, P. et al. Simultaneous painting of three genomes in hexaploid wheat by BAC-FISH. **Genome**, v. 47, n. 5, p. 979-87, 2004.

Appendixes

Appendix A – Markers used for *T. timopheevii* mapping

Markers of the A^t genome of *T. timopheevii* with the corresponded cM positions

Chr1At	cM	Chr2At	cM	Chr 3At	cM	Chr 4At	cM	Chr 5At	cM	Chr 6At	cM	Chr 7At	cM
AX-95239049	0	AX-95192332	0	AX-94927871	0	AX-94856207	0	AX-94511578	0	AX-94641398	0	AX-94595599	0
AX-94582997	10.9	AX-94804880	0.7	AX-94523332	0	AX-94433500	3.9	AX-94456236	0.7	AX-94527944	11.5	AX-94383872	1.5
AX-95163118	19.7	AX-95259382	0.8	AX-94542938	2.3	AX-94568929	8.5	AX-94899135	4.6	AX-94625380	23.1	AX-95198768	1.5
AX-95009775	26.0	AX-94538364	1.8	AX-94466419	4.5	AX-94397930	9.2	AX-94999947	10.8	AX-94786253	27.7	AX-94703949	2.2
AX-94836654	29.8	AX-95176938	3.0	AX-94832700	6.8	AX-94862616	12.3	AX-94846190	10.8	AX-94789654	30.7	AX-95221143	2.2
AX-94730241	30.5	AX-95257613	4.5	AX-94447907	9.0	AX-94945797	14.5	AX-95175117	10.8	AX-94627714	32.2	AX-94907830	3.0
AX-94393003	30.6	AX-95216598	7.1	AX-95220838	9.8	AX-94472289	17.5	AX-95097918	10.8	AX-95241582	35.2	AX-94880546	3.7
AX-94503609	33.6	AX-94695159	9.8	AX-95245143	10.5	AX-94479623	28.2	AX-95097417	11.6	AX-94821053	36.0	AX-94759638	3.7
AX-95014575	35.5	AX-94486514	9.8	AX-94783547	10.5	AX-95190358	38.8	AX-94434668	18.7	AX-94948332	37.5	AX-94938968	4.5
AX-94684631	37.3	AX-94896111	9.8	AX-94502353	10.5	AX-94855600	39.5	AX-94945628	26.6	AX-94706334	39.0	AX-94542816	6.7
AX-95151422	37.3	AX-94756243	11.3	AX-94852544	10.5	AX-94478005	40.3	AX-94430309	27.4	AX-94600202	46.1	AX-94383516	8.2
AX-94774946	37.3	AX-95008724	12.7	AX-94709618	11.2	AX-94784960	45.7	AX-95076236	27.4	AX-86179538	47.5	AX-95172377	8.2
AX-94522187	37.3	AX-95072207	15.5	AX-94873227	12.7	AX-94507064	51.6	AX-94723512	28.8	AX-95021534	48.3	AX-94502972	9.0
AX-94813794	37.3	AX-95223335	17.3	AX-94548447	14.2	AX-94576066	53.4	AX-94891640	28.8	AX-94625175	48.3	AX-94599101	9.7
AX-94510917	40.4	AX-94485513	17.3	AX-94900613	14.4	AX-94940770	54.2	AX-94745056	33.5	AX-94668789	48.3	AX-94494916	11.2
AX-94922849	43.4	AX-94906814	18.8	AX-94948041	15.0	AX-94476473	54.2	AX-94710091	43.2	AX-95251480	48.3	AX-94986725	12.7
AX-95134614	44.1	AX-94638064	20.2	AX-94612798	15.9	AX-94721591	62.1	AX-94749230	55.6	AX-94835245	49.0	AX-94630072	13.4
AX-94669313	45.6	AX-94799340	20.2	AX-94427362	18.7	AX-94942459	68.4	AX-94903992	55.6	AX-95225835	49.8	AX-94689244	13.4
AX-95141662	46.4	AX-94823845	21.7	AX-94471182	21.8	AX-95144713	69.9	AX-94392984	55.6	AX-94902458	49.8	AX-94469341	13.4

AX-86173170	46.4	AX-94386597	23.2	AX-94946799	22.5	AX-94529621	69.9	AX-94419595	57.1	AX-95236394	49.8	AX-95215247	13.4
AX-86178330	47.1	AX-94751668	24.7	AX-95003692	31.3	AX-94793000	72.2	AX-94399337	64.2	AX-94714460	55.2	AX-94565319	13.4
AX-94788006	47.1	AX-94573816	25.5	AX-95129979	40.1	AX-94554728	74.4	AX-95176087	66.5	AX-94605037	55.2	AX-95115516	13.4
AX-95083813	47.1	AX-94843132	26.2	AX-94664302	40.9	AX-94430995	74.4	AX-94773269	72.7	AX-95201485	61.5	AX-94695508	13.4
AX-94443776	47.8	AX-95080824	26.2	AX-94617990	40.9	AX-94750909	74.4	AX-94748288	72.7	AX-94639519	62.2	AX-94442494	13.4
AX-94526116	50.9	AX-94427350	28.5	AX-94856612	40.9	AX-94663483	74.4	AX-94479917	82.4	AX-94874011	67.6	AX-94416597	14.2
AX-94700094	53.1	AX-94528590	30.7	AX-95193187	42.4	AX-94578210	78.2	AX-94557327	90.4	AX-94466655	82.0	AX-95130767	14.9
AX-95122888	54.6	AX-94808162	30.7	AX-94417910	43.9	AX-94814284	82.1	AX-94497385	91.9	AX-94665597	82.8	AX-94437464	14.9
AX-95084016	55.4	AX-95115213	32.2	AX-94690364	44.6	AX-94716261	82.1	AX-94691847	91.9	AX-94881038	83.5	AX-94389087	14.9
AX-94591916	56.1	AX-94727794	32.2	AX-94621803	45.4	AX-95021715	82.8	AX-95123089	91.9	AX-94568819	84.2	AX-94694628	17.2
AX-94493329	56.8	AX-94412799	33.7	AX-95130498	45.4	AX-94492884	85.1	AX-95095532	94.1	AX-95023118	84.2	AX-95079348	19.4
AX-94777342	57.6	AX-95630135	33.7	AX-94484925	45.4	AX-94690629	89.7	AX-94494261	94.1	AX-94577446	84.2	AX-94391320	20.2
AX-94661112	57.6	AX-94672320	35.2	AX-94586703	46.1			AX-94489770	94.9	AX-94730838	84.2	AX-94538964	20.9
AX-94629293	57.6	AX-94394887	36.7	AX-94394183	46.8			AX-94381965	95.6	AX-94699879	93.1	AX-94389323	21.6
AX-94917775	57.6	AX-86165901	38.9	AX-94381828	46.8			AX-94429098	95.6	AX-94786019	97.7	AX-94839659	22.4
AX-94384347	57.6	AX-94504198	41.6	AX-94463669	47.6			AX-94483629	96.4	AX-94424299	97.7	AX-94779311	22.4
AX-95001661	58.3	AX-94391513	43.9	AX-94428990	47.6			AX-94622175	97.1	AX-95215678	97.7	AX-94976796	22.4
AX-95069046	59.1	AX-94828061	45.7	AX-94422817	48.3			AX-94398500	109.6	AX-95197676	97.7	AX-94417359	24.6
AX-94439447	59.1	AX-94911937	45.7	AX-94520083	49.8			AX-94897313	113.4	AX-95199148	97.7	AX-94787236	26.9
AX-94714481	59.1	AX-94462589	45.7	AX-94861433	51.3			AX-94445171	119.6	AX-94910882	97.7	AX-94682902	26.9
AX-94731169	59.1	AX-95167337	45.7	AX-94398068	52.0			AX-94452971	125.9	AX-95138423	97.7	AX-94505780	26.9
AX-94456521	59.1	AX-94678573	45.7	AX-94433258	52.8			AX-94905515	130.5	AX-94980915	97.7	AX-94751167	26.9
AX-95207074	59.1	AX-95184497	46.5	AX-94928542	52.8			AX-94419987	140.2	AX-94563936	97.7	AX-95189111	26.9
AX-94636953	61.3	AX-95163698	47.9	AX-95015701	52.8			AX-94890412	162.0	AX-94746124	97.7	AX-94387074	31.5
AX-95138427	63.6	AX-94553665	50.2	AX-95075588	52.8					AX-94619757	97.7	AX-94972900	31.5
AX-94859014	63.6	AX-94666225	54.8	AX-95216392	53.5					AX-94674861	97.7	AX-94638909	32.3

AX-94775110	63.6	AX-94394578	60.3	AX-95123927	53.5
AX-95082893	64.3	AX-95188312	62.5	AX-94852279	54.4
AX-94461267	65.1	AX-94423793	64.8	AX-94866868	55.2
AX-94993961	65.1	AX-94636452	64.8	AX-94495556	58.8
AX-94870541	65.1	AX-94697318	66.3	AX-95234197	61.8
AX-94490465	65.1	AX-95131782	66.3	AX-94541427	65.7
AX-94400275	65.1	AX-94409581	67.8	AX-94688017	68.7
AX-94411374	65.1	AX-95183011	68.9	AX-94812241	69.4
AX-94694335	65.1	AX-94460418	70.0	AX-94995240	73.3
AX-95182380	65.8	AX-94623199	71.5	AX-94983046	79.3
AX-94905532	66.5	AX-94613797	72.6	AX-94568176	80.9
AX-94778336	66.5	AX-94657384	73.7	AX-95006927	81.7
AX-94528863	66.5	AX-94464916	74.4	AX-94771453	83.2
AX-94619761	66.5	AX-95169440	75.2	AX-94493067	83.2
AX-94478936	69.6	AX-94818415	80.3	AX-95218076	84.0
AX-94620755	69.6	AX-95136834	85.0	AX-94720408	84.0
AX-94862780	73.4	AX-94488606	86.7	AX-95201257	84.4
AX-94978926	77.2	AX-94921756	86.7	AX-94813670	84.7
AX-94530188	77.2	AX-95129278	89.0	AX-94831084	84.9
AX-94809993	77.9	AX-94470676	89.7	AX-95070910	85.1
AX-94590927	82.6	AX-94872303	90.5	AX-94829627	85.2
AX-94498696	84.8	AX-94427576	92.7	AX-94663948	85.4
AX-95180111	90.7	AX-94992596	95.0	AX-94485226	86.2
AX-94990974	94.4	AX-95168254	97.2	AX-94678869	87.7
AX-94473649	101.2	AX-94459270	97.2	AX-86176361	88.4
AX-95005100	105.0	AX-94941367	99.5	AX-95081840	94.7
AX-94598684	100.7	AX-94602262	35.3		
AX-94433552	100.7	AX-94486555	35.3		
AX-94560350	100.7	AX-95094492	35.3		
AX-94472595	100.7	AX-95248497	35.3		
AX-94776871	100.7	AX-95120206	37.5		
AX-94536138	103.0	AX-94837407	37.5		
AX-94563888	103.0	AX-94434698	37.5		
AX-95204598	103.0	AX-94522885	37.5		
AX-94934454	103.0	AX-94912175	37.5		
AX-94618325	103.0	AX-94384726	39.0		
AX-94488716	103.0	AX-94470543	40.0		
AX-95220252	103.0	AX-86176910	42.0		
AX-94468806	103.0	AX-94547905	44.3		
AX-95189832	103.0	AX-94436934	45.0		
AX-95100089	103.7	AX-94495956	52.1		
AX-94457159	103.7	AX-94468226	52.1		
AX-95025699	109.1	AX-94986625	55.2		
AX-94863938	109.1	AX-94889187	55.2		
AX-94926949	123.5	AX-94747132	57.4		
AX-94735786	125.0				

AX-94982575	105.0	AX-94742725	108.3	AX-95024572	94.7
AX-95093988	105.0				
AX-95202547	105.0				

Markers of the G genome of *T. timopheevii* with the corresponded cM positions

Chr1G	cM	Chr 2G	cM	Chr 3G	cM	Chr 4G	cM	Chr 5G	cM	Chr 6G	cM	Chr 7G	cM
AX-94460938	0	AX-94828386	0	AX-94519442	0	AX-86167869	0	AX-95015550	0	AX-95256407	0	AX-95159346	0
AX-94711679	0.7	AX-94698374	0.7	AX-94470995	0	AX-94603489	0	AX-94833651	0.7	AX-94745890	0	AX-95223512	0.7
AX-94645609	0.7	AX-94463791	10.4	AX-94792222	0	AX-95162453	0	AX-95158641	0.7	AX-95102445	2.3	AX-94711978	0.7
AX-94524812	2.2	AX-94897568	34.5	AX-94655585	0	AX-95241638	0	AX-94637263	0.7	AX-94905905	6.9	AX-94657427	0.7
AX-94885606	4.5	AX-94793912	34.5	AX-94743899	0	AX-95130149	0	AX-95102702	0.7	AX-95142186	14.8	AX-95190773	1.5
AX-94418372	5.2	AX-94517516	38.4	AX-94412171	0.7	AX-95232028	1.5	AX-95210844	0.7	AX-94414937	21.1	AX-95080006	3.0
AX-94657479	6.7	AX-94601079	39.1	AX-94523038	0.7	AX-94589541	1.5	AX-94610861	0.7	AX-95122886	21.8	AX-94608702	4.5
AX-94757643	8.2	AX-94649867	39.1	AX-95186328	0.7	AX-94824449	3.0	AX-94999851	0.7	AX-95010034	25.6	AX-95239692	4.5
AX-94802682	9.0	AX-94896890	43.7	AX-95127154	2.2	AX-94663013	4.5	AX-94499088	0.7	AX-95198819	26.4	AX-94924124	6.0
AX-94700304	9.7	AX-94628011	48.3	AX-95231401	3.0	AX-94744972	4.5	AX-95022135	0.7	AX-95070251	27.1	AX-94973270	7.5
AX-94683105	9.7	AX-94491351	48.3	AX-94840232	3.7	AX-95005286	6.0	AX-95196211	0.7	AX-94833113	29.4	AX-94945900	7.5
AX-94762865	10.5	AX-94790617	48.3	AX-94759675	3.7	AX-94995879	7.5	AX-95141719	0.7	AX-94813039	30.1	AX-94541333	8.9
AX-94415591	11.2	AX-94642607	48.3	AX-94739231	6.0	AX-95189434	17.2	AX-95149626	3.0	AX-94864700	30.9	AX-94574308	10.4
AX-95116095	11.2	AX-94884817	48.3	AX-94525262	7.5	AX-94661544	17.2	AX-94390361	3.0	AX-94404996	31.6	AX-94485553	10.4
AX-94561589	11.2	AX-95204758	48.3	AX-95002760	7.5	AX-94519609	18.7	AX-94731801	3.0	AX-94446593	38.7	AX-94400417	11.9
AX-94585755	11.2	AX-94510765	49.1	AX-95114368	7.5	AX-94646391	20.2	AX-95229293	3.0	AX-95118964	46.7	AX-94886229	11.9
AX-95154690	13.5	AX-95142559	49.8	AX-94392142	7.5	AX-94797205	20.2	AX-95179297	3.0	AX-94664517	57.3	AX-94534396	11.9
AX-94614192	15.7	AX-94477217	49.8	AX-95102903	8.2	AX-95005995	21.6	AX-94438942	3.0	AX-94697250	75.7	AX-94517409	11.9
AX-94460759	16.4	AX-94742600	66.2	AX-94773596	8.2	AX-94769935	21.6	AX-94596869	3.0	AX-94802770	80.4	AX-94477496	11.9
AX-95255108	17.2	AX-95225623	66.2	AX-95241340	8.2	AX-94834923	23.1	AX-94644008	3.0	AX-94988458	81.1	AX-94893631	13.4
AX-95188674	17.2	AX-94802300	66.2	AX-94588325	8.9	AX-94603548	24.6	AX-94797492	3.0	AX-94404191	82.6	AX-94523935	15.7
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AX-94690295	69.2
AX-95108183	69.2
AX-94791652	69.2
AX-94416047	69.2
AX-94928608	69.2
AX-94638838	69.2
AX-94761974	78.9
AX-94805775	78.9
AX-94382232	79.7
AX-94557592	79.7
AX-94600934	79.7
AX-94614828	79.7
AX-95126627	79.7
AX-94390156	79.7
AX-94746152	79.7
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AX-94471407	32.1
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AX-94461707	32.1
AX-94456002	32.1
AX-95240372	32.1
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AX-94649106	32.1
AX-94671630	32.1
AX-94412685	32.1
AX-95194904	36.7
AX-95007739	36.7
AX-95117413	38.2
AX-94983233	38.2
AX-94428806	38.2
AX-94724199	42.8
AX-94399413	42.8
AX-94748768	42.8
AX-94617079	42.8
AX-94650553	42.8
AX-94573573	44.3
AX-95208581	44.3
AX-95169718	44.3
AX-94915329	44.3
AX-94749233	44.3
AX-94691860	44.3
AX-95228735	44.3

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AX-94680690	79.7
AX-94990750	79.7
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AX-94913838	79.7
AX-94587803	79.7
AX-94554755	79.7
AX-94446606	79.7
AX-94937792	79.7
AX-94441780	79.7
AX-94868919	79.7
AX-94774515	79.7
AX-86176865	79.7
AX-95123223	85.1
AX-94413948	85.1
AX-94554552	85.1
AX-94789346	85.1
AX-94933159	85.1
AX-94805502	85.1
AX-86184819	85.1
AX-94436745	85.1
AX-94565831	86.6
AX-95220774	88.1
AX-95231491	88.1
AX-94394454	88.1
AX-94777098	44.3
AX-95242385	44.3
AX-94637076	44.3
AX-94878787	44.3
AX-95107195	45.1
AX-94656806	45.1
AX-94557518	45.1
AX-95178859	45.1
AX-95163981	45.1
AX-95137082	45.1
AX-86177094	45.1
AX-94737123	45.1
AX-95168832	45.1
AX-94790093	45.1
AX-95001464	45.1
AX-94501718	45.1
AX-94473466	45.1
AX-95132630	45.1
AX-95115211	45.1
AX-95157769	45.8
AX-94696479	45.8
AX-94893504	45.8
AX-95080756	45.8
AX-94926022	48.1
AX-94633885	48.1
AX-94727436	48.1
AX-94852732	48.1

AX-86179234	88.1
AX-94430439	88.1
AX-95124055	88.1
AX-95220018	88.1
AX-94734382	88.1
AX-94417218	88.1
AX-94459718	88.1
AX-95630025	88.1
AX-86165433	88.1
AX-95189895	88.1
AX-94466347	88.1
AX-94642747	88.1
AX-94593471	90.4
AX-94410588	108.8
AX-94427642	112.6

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AX-94973664	54.3
AX-94651710	54.3
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AX-94868420	54.3
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AX-94491624	55.1
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AX-95178296	55.1
AX-94764219	55.1
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AX-94571628	56.5
AX-95215143	56.5
AX-94780862	56.5
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AX-94508002	57.3
AX-94430374	58.8
AX-94705918	58.8
AX-94919470	58.8
AX-94613238	58.8
AX-94566857	58.8
AX-94922814	62.6
AX-94572247	62.6
AX-94925625	62.6
AX-94570956	90.4
AX-94901051	94.2

Attachments

Attachment A – Protocols

Nick translation reaction 20 µL in 1.5 mL tube:	**Amount**
DNA (gDNA > 200 ng μL^{-1})	2 - 3 µg
10 x Nick translation buffer or 10 x buffer2	2.0 µL
Non-labeled dNTPs (2 mM each, mixed)	2.0 µL
Labeled dNTP (1 mM)	0.5 µL
DNA polymerase I (10 U/ µL)	5.0 µL
DNase (100 mU/ µL) diluted (prepare just before using) (5 µL of 2 U/µL DNase add 95 µL of 50 % glycerol)	0.8 µL

Mix well by pipetting.

Incubate at 16 °C for 2 h in dark or covered by foil.

Ethanol Precipitation of the probe

1. Add 8 x of the reaction volume of fragmented (autoclaved) 140 ng μL^{-1} SS DNA working solution: 160 µL (over 20 ng SS DNA). Vortex.

2. Add 25 x of the reaction volume of 9:1 (V/V 100 % ethanol: 3 M sodium acetate pH 5.2) precipitation solution: 500 µL, mix well.

3. Incubate at - 20 °C overnight

4. Spin at 12,000 rpm for 30 min at 4 °C

5. Wash the probe pellet with 70 % ethanol; spin down for 5 min.

Repeat 70 % ethanol wash; spin down, discard ethanol.

6. Air-dry the probe in dark for 5 -10 min. Make sure no ethanol left.

7. Dissolve the pellet in 20 µL of 2 x SSC + 1 x TE buffer pH 7.6 (~200 ng μL^{-1}) on ice (4 °C) in dark for 30 min - 1 h, mix by tapping the tube and spin down briefly.

8. Store at - 20 °C.

Preparation of blocking genomic DNA

Aliquot 500 ~ 700 µL of gDNA (~2 µg μL^{-1} in TE or dH_2O) into a 1.5 mL tube, seal its cap tightly with NASCO film and treat the DNA by **one** of the ways:

1. in boiling water for 30 - 40 min;
2. in 100 - 110 ◦C heat block for 15 min;
3. in a pressure cooker at 115 ◦C for 8 min.

Check the sizes of DNA on a gel. The DNA should be broken into 300 bp to 500 bp. Store the blocking DNA at – 20 °C.

Preparation of Enzyme solution	**Amount**
To make 10 g of the enzyme solution on ice:	
Pectolyase Y-23 (1 % w/w)	0.1 g
Cellulase Onozuka R-10 (2 % w/w)	0.2 g
1X Citric Buffer (pH 5.5)	9.7 g

Mix above and dispense 15 - 20 µL aliquot into each 0.5 mL tube on ice and store at - 20 °C.

Citric Buffer Stock	**Amount**
5x Citric Buffer pH 5.5	50 mL
dH_2O	30 mL
50 mM trisodium citrate, dihydrate $C_6H_5O_7Na_3 \cdot 2H_2O$ FW = 294.12)	0.735 g
50 mM EDTA 0.5M	5 mL

Adjust the solution to pH 5.5 using citric acid monohydrate
($C_6H_8O_7 \cdot H_2O$ FW = 210.14)

Add the solution with dH_2O up to 50 mL; dissolve and mix well.

Sterilize the solution by using syringe with filter and store at RT/Fridge.

Preparation of metaphase spreads from root tips

[Nitrous oxide-enzymatic maceration method (Kato, 1999) modified]

1. Germinate seeds for 2 - 3 days on wet tissue when roots grow 2 - 3 cm long.

2. Make a 0.5 mL tube with a hole on the lid, spread a thin lay of dH_2O to the opening tube, put the wet tube on ice

3. Cut off 1 - 3 roots about 1 cm in length, put them into the tube immediately.

4. Put all sample tubes in the Nitrous oxide sample chamber; close it properly and connect it to the Nitrous oxide gas unit.

5. Release Nitrous oxide gas at 10 bar for 30 s to 1 min; turn off the gas to the chamber, release the pressure between the connected part and disconnected the gas unit from the sample chamber.

6. Keep the samples in the chamber with N_2O for 2 h. Release the gas pressure and put the samples on ice.

7. Fill the sample tube with 90 % acetic acid to fix the treated roots for at least 10 min on ice.

8. Pipette out the acetic acid using a glass pipette; fill the tube with dH_2O and wash the roots with dH_2O three times by pipetting up and down (make them dance and leave them soaking a few minutes in between washes) **with care** to avoid the root missing.

    - If the roots will be stored for late use, then transfer the roots into a new 0.5 mL tube with ~150 µL of 70 % ethanol. Store the sample tube in the storage box at – 20 °C.

      BEST TO CARRY STRAIGHT ON IF YOU HAVE TIME.

    - Wash the roots with dH_2O three times (at least 10 min per wash; better keep in the last dH_2O for 0.5 h or so); make sure no any ethanol in the root tips before using enzyme for preparation of metaphase spreads:

9. Put the roots on a filter paper, remove extra dH_2O but do not dry them out.

10. Cut the root tip ~ 1 mm in length (white bit) which contains the active dividing region and transfer the root tip into a 0.5 mL tube containing ~ 20

µL of enzyme solution (Pectolyase 1 % and Cellulase 2 %).

11. Incubate at 37 °C for 45 - 50 min depending on sizes and species of the roots (need to be optimized first), then put the sample tubes on ice.

12. Add 150 µL of 70 % ethanol to wash off the enzyme from the root tips 2 times with care (again make them dance in the ethanol to wash off all the enzyme); and keep the root tips in ~100 µL of 70 % ethanol on ice.

13. Break (press) the root tips with a bit ethanol against the tube wall into very fine cell suspension with a dissection needle carefully.

14. Tap the tube genteelly several times to suspend the cells.

15. Centrifuge the tube at 5000 rpm for 30 s and remove the ethanol carefully; do not disturb the cell pellet.

16. Dry the cell pellet briefly on a piece of tissue on the table with the lid open (a few minutes, do not over dry!), add 25 - 40 µL (dependent on size of pellet) of 100 % acetic acid; keep the tube on ice for 10 min to 2 h.

17. Prepare a cardboard box laid with a few layer of wet tissue on a water proof film and put the labelled slides on a plastic supporter

18. Vortex the tube briefly, take 6 - 7 µL of cell suspension and drop on to a slide at the distance above the slide 5 – 10 cm,

19. Close the cardboard box and let the slide gradually drying out for over 10 min.

GISH and Multi-color GISH detections (Avoid direct light)

Selected chromosome spread slides and circle the sample area on other side of slide by using mark pen; place the slide with chromosome spread up on a slide holder

Treat the slide by UV light at 0.125 Joules two times for cross link the chromatins on the slide.

Single probe- GISH

Probe: labeled gDNA with Alexa Fluor-488 (Green) 5-dUTP or other fluorophores

AA BB DD genome blocking gDNA - CS gDNA fragmented (300 bp - 500 bp)

GISH Probe mixture	**10 µL per slide**
2 x SSC in 1x TE	X µL up to 10 µL
Probe gDNA(green) (100 ng μL^{-1})	0.5 - 1 µL

Blocking gDNA (fragmented 1000 ng μL^{-1}) 2 - 4 µL

(added blocking DNA needs over 20 – 40 x of the probe DNA)

Mix by pipetting (avoid air-bubbles) and carry on hybridisation .

Hybridisation

1. UV Cross link the slides at 0.125 Joules for 2 times.
2. Heat up half container of deionized water covered with foil by induction hot plate.
3. Add the probe mix onto the cross linked samples.
4. Cover with a plastic cover slip (22 mm x 22 mm), avoid air bubbles.
5. Place the slide on wet tissue firmly in a stainless tray and cover the slides with a tip box lid.
6. Switch off the hob when the water is boiling, then put the slide tray in and covered the whole container with foil for 5 min.
7. Place the denatured the slide on slide holder in a container with wet tissue, closed with a lid.
8. Incubate the slide container at 55 °C for overnight.
9. Dip the slide into 2 x SSC to make the cover slip fall off from the slide. Use blue roll on the side of slides to absorb excess 2 x SCC and steam from the incubator overnight.
10. Add a drop of Vectashield mounting medium with DAPI (or 1:2 diluted with 1 x PBS), put a 24 x 50 mm glass cover slip carefully-avoid avoid air bubbles. Again allow to dry/use blue roll on edges to absorb excess liquid before viewing/storage.
11. Observe with multi-filter microscopy for DAPI, Alexa Fluor-488 (Green) and Alexa Fluor-594 (Red)**.**

Vitae

Mariana Peil da Rosa nasceu em 16 de janeiro de 1988, em Pelotas – RS, filha de Maria Cristina Machado Peil (advogada) e Carlos Alberto Leite da Rosa (administrador). Em 2007 ingressou no curso de Agronomia, na Faculdade de Agronomia Eliseu Maciel – UFPel, concluindo a graduação no segundo semestre de 2011. Durante a faculdade realizou um de estágio voluntário na área de plantas ornamentais, sob orientação do Prof. PhD Paulo Roberto Grolli, após foi estagiar no Laboratório de Ciência e Tecnologia de Sementes, um como voluntária, sob orientação do Prof. PhD, Silmar Peske, e um como bolsista de iniciação científica CNPq, sob orientação do Prof. PhD Leopoldo Baudet. No último ano de graduação estagiou no Laboratório de Propagação de Frutíferas de Clima Temperado, sob orientação da Prof. Dr. Márcia Schuch. No segundo semestre de 2011 realizou seu estágio final com tratamento de sementes de trigo e cevada visando tolerância à estresses abióticos no "*Agricultural Research Organization – Volcani Center*" uma instituição de pesquisa governamental em Bet Dagan – Israel, a orientação acadêmica foi do Prof. Dr. Silmar Peske e a orientação profissional do PhD Joshua Klein. Em março de 2012, ingressou no curso de mestrado, com bolsa da CAPES, no PPG em Ciência e Tecnologia de Sementes, cujo foco da dissertação foi a expressão de genes em tegumento de sementes de soja, sob orientação do Prof. Dr. Paulo Dejalma Zimmer. No segundo semestre de 2014, ingressou no curso de doutorado no PPG em Agronomia, na área de concentração de Fitomelhoramento, com bolsa da CAPES, sob orientação do Prof. PhD. Antonio Costa de Oliveira. No segundo semestre de 2015 até metade de 2016, realizou estágio de doutorado *sandwich* na *The University of Nottingham* – Reino Unido, onde realizou o trabalho de sua tese. Em março de 2018 defendeu sua qualificação com o "O uso de CRISPR como ferramenta para controle em cromossômico em cromossômico em *Triticum aestivum".*