

**The development of introgression lines of  
*Triticum timopheevii* in a cultivated wheat  
background and their characterisation**

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## Abstract

Achieving a sustainable supply of food in the face of the rise in the global population and climate change is a major challenge for plant breeders. Crop improvement through breeding is only possible where genetic variation is present in the breeder's germplasm, but decades of intensive selection in major crops has inevitably narrowed their genetic diversity.

The efficient use of the biodiversity of wild relatives of wheat is key for the enhancement of genetic variability. Many wild relatives of wheat possess a vast potential reservoir of genetic variation for abiotic and biotic stresses e.g. drought tolerance, resistance to insects and fungal diseases, biomass, yield and photosynthetic potential.

*Triticum timopheevii* is a tetraploid wheat ( $2n=28$ ,  $A^tA^tGG$ ), which is characterized by complex resistance genes to many diseases. Genes for resistance to stem rust (*Sr36*), powdery mildew (*Pm2*, *Pm6*, *Pm27*) and leaf rust (*Lr18*) have been exploited. Hence, it has been used in this programme with the prime objective of developing chromosome introgression lines for the  $A^t$  and G genomes of *T. timopheevii* in bread wheat. Characterization of wheat/*T. timopheevii* introgression lines was carried out via an Axiom® 35K SNP wild relative array. A putative linkage map of *T. timopheevii* has been successfully developed and confirmed by exploitation of fluorescence *in situ* hybridization (FISH). Development of wheat chromosome specific KASP markers which will aid in further characterization of the introgressions is underway.

*T. timopheevii* accession assessed for mineral concentrations demonstrated variation which can be further exploited in wheat breeding micronutrients. Variations for Fusarium head blight (FHB) in accessions of *T. timopheevii* were also demonstrated and mapping populations developed to map the resistance genes for FHB. The results show the presence of novel type I resistance present in *T. timopheevii*. Mapping of FHB resistance genes with KASP markers is underway.

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## List of Abbreviations

| Abbreviation                    | Description                                  |
|---------------------------------|----------------------------------------------|
| GPC                             | Grain protein content                        |
| BP                              | Before present                               |
| <i>Acc-1</i>                    | Acetyl-coA carboxylase                       |
| <i>Pgk-1</i>                    | 3-phosphoglycerate kinase                    |
| <i>Ph1</i>                      | Pairing homoeologous 1                       |
| <i>Lr</i>                       | Leaf rust                                    |
| <i>Sr</i>                       | Stem rust                                    |
| <i>Pm</i>                       | Powdery mildew                               |
| <i>Yr</i>                       | Yellow rust                                  |
| <i>Gc</i>                       | Gametocidal genes                            |
| ISH                             | <i>in situ</i> hybridisation                 |
| GISH                            | Genomic <i>in situ</i> hybridisation         |
| FISH                            | Fluorescence <i>in situ</i> hybridisation    |
| mcGISH                          | Multicolour GISH                             |
| NOR                             | Nucleolus organizing regions                 |
| RFLPs                           | Restriction Fragment Length Polymorphisms    |
| RAPDs                           | Randomly Amplified Polymorphic DNAs          |
| AFLPs                           | Amplified Fragment Length Polymorphisms      |
| SSR                             | Simple Sequence Repeats                      |
| SNP                             | Single Nucleotide Polymorphism               |
| ESTs                            | Expressed Sequence Tags                      |
| COS                             | Conserved Orthologous Sequences              |
| STS                             | Sequence Tagged Sites                        |
| DArT                            | Diversity array technology                   |
| KASP                            | Kompetitive allele-specific PCR              |
| GBS                             | Genotyping-by-Sequencing                     |
| MAS                             | Marker assisted selection                    |
| cM                              | CentiMorgan                                  |
| Kb                              | Kilobases                                    |
| Fe                              | Iron                                         |
| Zn                              | Zinc                                         |
| FHB                             | Fusarium head blight                         |
| HMW                             | High molecular weight                        |
| CINaO                           | Sodium hypochlorite                          |
| DNA                             | Deoxyribonucleic acid                        |
| dNTPs                           | Deoxyribo-nucleotide triphosphates           |
| SSC                             | Saline-sodium citrate                        |
| PFA                             | perfluoroalkoxy                              |
| PEEK                            | polyethylethylketone                         |
| CRM                             | Certified reference material                 |
| LRM                             | Laboratory reference material                |
| ICP-MS                          | Inductively coupled plasma mass spectrometry |
| KH <sub>2</sub> PO <sub>4</sub> | Potassium phosphate                          |
| K <sub>2</sub> SO <sub>4</sub>  | Potassium sulfate                            |
| H <sub>3</sub> BO <sub>3</sub>  | Boric acid                                   |
| <i>Pina</i> and <i>Pinb</i>     | Puroindoline a and b                         |
| GBBSI                           | Granule-bound starch synthase I              |
| EMS                             | Ethyl methanesulfonate                       |
| Y                               | Gamma                                        |
| CS                              | Chinese spring                               |
| QTL                             | Quantitative trait locus                     |
| APT                             | Affymetrix powertools                        |
| ADAP                            | Axiom® Data Analysis pipeline                |



|        |                                                           |
|--------|-----------------------------------------------------------|
| PHR    | Polymorphic high resolution                               |
| MHR    | Monomorphic high resolution                               |
| CRBT   | Call rate below threshold                                 |
| LOD    | Log-odds                                                  |
| IWGSC  | The International Wheat Genome Sequence Consortium        |
| GGT    | Graphical Genotypes                                       |
| PCR    | Polymerase chain reaction                                 |
| TGAC   | The Genome Analysis Centre                                |
| dGTP   | Deoxyguanosine triphosphate                               |
| NRGene | Next Generation Complex Genome Assembly                   |
| PoPSEQ | Population sequencing                                     |
| LG     | Linkage group                                             |
| CGIAR  | Consultative group on International Agricultural Research |
| SPAD   | Soil-Plant Analysis Development                           |
| DON    | deoxynivalenol                                            |
| ZEA    | Zearalenone                                               |
| PRISM  | Portable Reusable Integrated Software Modules             |
| Cv.    | Cultivar                                                  |

# **Chapter 1**

## **Introduction and literature review**

### **1.1 Introduction**

The global production of bread wheat in 2014 was 725 million tonnes, with an average yield of 3t/ha (FAO, 2015). It was grown on 217 million hectares throughout the world (FAO, 2008a) and in particular it was grown on 57 million hectares with 190 million tonnes grain harvested in Europe. World demand for wheat is continually increasing with estimates indicating a requirement for a 60% increase in wheat production by 2050 (Ray *et al.*, 2013). Wheats are extensively grown across the temperate, Mediterranean, and subtropical parts of both hemispheres of the world. The world's major bread wheat-producing areas are in northern China, northern India, northern USA and adjoining areas in Canada, northern and central Europe, western Russia, southern Australia, southern Latin America and South Africa.

Wheat is the most important cereal crop for the majority of the world's population as it is the staple food in the ancient and the modern world not only for billions of people, but also for animal feed, occupying 17% of the crop acreage worldwide. A total of 95% of the daily breads, cakes and pastries that humans consume come from bread wheat, while the remaining 5% come from tetraploid durum wheat (Dubcovsky and Dvorak, 2007). About 40% of the world's population uses wheat as feed and it provides 20% of the total food calories and protein in human nutrition as well as providing nearly 55% of the carbohydrates (Breiman and Graur, 1995). Wheat is used to produce starch, malt, dextrose, gluten, alcohol and other products (Nevo *et al.*, 2002). Due to the high content of starch in wheat, it is an attractive option as a "first

generation biofuel" (saccharification) which can then be fermented into ethanol (Erdei *et al.*, 2010).

## **1.2 Taxonomy and morphology of wheat**

Wheat belongs to the grass family Poaceae, subfamily Pooideae and tribe Triticeae (=hordeae) (Briggle and Reitz, 1963). The Poaceae includes many important crops with diverse native distributions with at least a 35-fold variation in genome size (Paterson *et al.*, 2005). The tribe Triticeae consists of two genus, *Triticum* and *Aegilops* (van Slageren, 1994).

The genus *Triticum* consists of six species: *Triticum monococcum* (AA genome), *Triticum urartu* (AA), *Triticum turgidum* (AABB), *Triticum timopheevii* ( $A^tA^tGG$ , where  $A^t$  is A genome of *T. timopheevii*), *Triticum aestivum* (AABBDD) and *Triticum zhukovskyi* ( $A^mA^mA^tA^tGG$ ,  $A^m$  is A genome of *T. monococcum*). These species are grouped into three sections as shown in Table 1.1, namely diploid species ( $2n=2x=14$ ); tetraploid species ( $2n=4x=28$ ) and hexaploid species ( $2n=6x=42$ ). *T. urartu* exists only in its wild form, whereas *T. aestivum* and *T. zhukovskyi* exist only as cultivated forms. *T. monococcum*, *T. turgidum* and *T. timopheevii* have both a wild and a domesticated form. The genus *Aegilops* (goatgrass) belongs to the tribe Triticeae and comprises 11 diploid, 10 tetraploid and two hexaploid species (van Slageren, 1994) where the U, M, S and C genomes were identified (Kilian *et al.*, 2011).

**Table 1.1:** Species belonging to the genus *Triticum* and *Aegilops*

| Species            | Genome                          | Triticum                                         | Genome                        | Aegilops               |
|--------------------|---------------------------------|--------------------------------------------------|-------------------------------|------------------------|
| <b>Diploids</b>    | A <sup>m</sup>                  | <i>T. monococcum</i>                             | S                             | <i>Ae. speltoides</i>  |
|                    |                                 | <i>T. boeoticum</i>                              |                               |                        |
|                    | A                               | <i>T. urartu</i>                                 |                               |                        |
|                    |                                 |                                                  | S <sup>l</sup>                | <i>Ae. longissima</i>  |
|                    |                                 |                                                  | S <sup>sh</sup>               | <i>Ae. sharonesis</i>  |
|                    |                                 |                                                  | S <sup>s</sup>                | <i>Ae. searsei</i>     |
|                    |                                 |                                                  | S <sup>s</sup>                | <i>Ae. bicornis</i>    |
|                    |                                 |                                                  | D                             | <i>Ae. tauschii</i>    |
|                    |                                 |                                                  | T                             | <i>Ae. mutica</i>      |
|                    |                                 |                                                  | M                             | <i>Ae. comosa</i>      |
|                    |                                 |                                                  | C                             | <i>Ae. caudata</i>     |
|                    |                                 |                                                  | U                             | <i>Ae. umbellulata</i> |
|                    |                                 |                                                  | N                             | <i>Ae. uniaristata</i> |
| <b>Tetraploids</b> | AB                              | <i>T. turgidum</i>                               | D <sup>c</sup> C <sup>c</sup> | <i>Ae. cylindrica</i>  |
|                    |                                 | <i>var. durum</i>                                |                               |                        |
|                    |                                 | <i>var. dicoccum</i>                             |                               |                        |
|                    |                                 | <i>var. carthlium</i>                            |                               |                        |
|                    |                                 | <i>var. dicoccoides</i>                          |                               |                        |
|                    | A <sup>t</sup> G                | <i>T. araraticum</i>                             | D <sup>v</sup> N <sup>v</sup> | <i>Ae. ventricosa</i>  |
|                    |                                 | <i>T. timopheevii</i> ssp.<br><i>timopheevii</i> |                               |                        |
|                    |                                 |                                                  | UC <sup>t</sup>               | <i>Ae. triuncialis</i> |
|                    |                                 |                                                  | UM                            | <i>Ae. geniculata</i>  |
|                    |                                 |                                                  | UM                            | <i>Ae. neglecta</i>    |
|                    |                                 |                                                  | UM                            | <i>Ae. columnaris</i>  |
|                    |                                 |                                                  | UM                            | <i>Ae. biuncialis</i>  |
|                    |                                 |                                                  | US <sup>t</sup>               | <i>Ae. kotschyi</i>    |
|                    |                                 |                                                  | US <sup>t</sup>               | <i>Ae. peregrina</i>   |
| <b>Hexaploid</b>   | ABD                             | <i>T. aestivum</i>                               | DMU                           | <i>Ae. juvenalis</i>   |
|                    |                                 | <i>var. spelta</i>                               |                               |                        |
|                    |                                 | <i>var. compactum</i>                            |                               |                        |
|                    |                                 | <i>var. sphaerococcum</i>                        |                               |                        |
|                    |                                 | <i>var. vavilovii</i>                            |                               |                        |
|                    |                                 | <i>var. macha</i>                                |                               |                        |
|                    | A <sup>t</sup> A <sup>m</sup> G | <i>T. zhukouskyi</i>                             | DDM                           | <i>Ae. crassa</i>      |
|                    |                                 |                                                  | UMN                           | <i>Ae. neglecta</i>    |
|                    |                                 |                                                  | DMS                           | <i>Ae. vavilovii</i>   |

Note: there is still debate for some species if they are still in variation or not

Mutations played a major role in the domestication of wheat giving rise to different morphological changes (Simons *et al.*, 2006). The shift from wild diploid and tetraploid genotypes to cultivated genotypes of hexaploid wheat includes changes in morphological characters related to seed dispersal. These changes have included spike dimensions (Huskins, 1946; Sears, 1952, 1954;

Simons *et al.*, 2006), spike rachis fragility (Leighty and Boshnakian, 1921; Mackey, 1954; Singh *et al.*, 1957; Jantasuriyarat *et al.*, 2004), spike length and plant height (Muramatsu, 1963; Kato *et al.*, 1999, 2003), pubescence (Kato *et al.*, 1999), grain size and glume tenacity (Muramatsu, 1963, 1986; Singh, 1969) and threshability (Kerber and Rowland, 1974; Simons *et al.*, 2006).

The genetic changes responsible for the suite of traits that differentiate domesticated plants from their wild ancestor are referred to as the domestication syndrome (Dubcovsky and Dvorak, 2007). In wheat the primary component of domestication was the loss of spike shattering, preventing the grains from being scattered by wind and facilitating harvesting. The chromosome locations of the brittle rachis (*Br*) genes controlling shattering in emmer wheat were found to be on chromosomes 3A and 3B (Levy and Feldman, 1989a; Watanabe *et al.*, 2002; Millet *et al.*, 2013).

Genotypes with soft glumes that require limited mechanical action during the dehulling process are considered free-threshing (Jantasuriyarat *et al.*, 2004). Bread wheat, with the exception of *T. aestivum* (var. *spelta* and var. *macha*), has tough inflorescence stems that do not shatter when harvested and the seeds are easily threshed after gathering (Simons *et al.*, 2006; Hancock, 2004).

Another important trait for wheat domestication was the loss of the tough glumes, converting hulled wheat into free threshing wheat. The prime genetic determinants of the free-threshing habit were recessive mutations at the *Tg* loci (tenacious glumes), accompanied by modifying effects of the dominant mutation at the *Q* locus and modifications at several other loci (Kerber and Rowland, 1974). The *Q* gene influences many other domestication related traits such as glume shape and tenacity, rachis fragility, plant height, spike length and ear

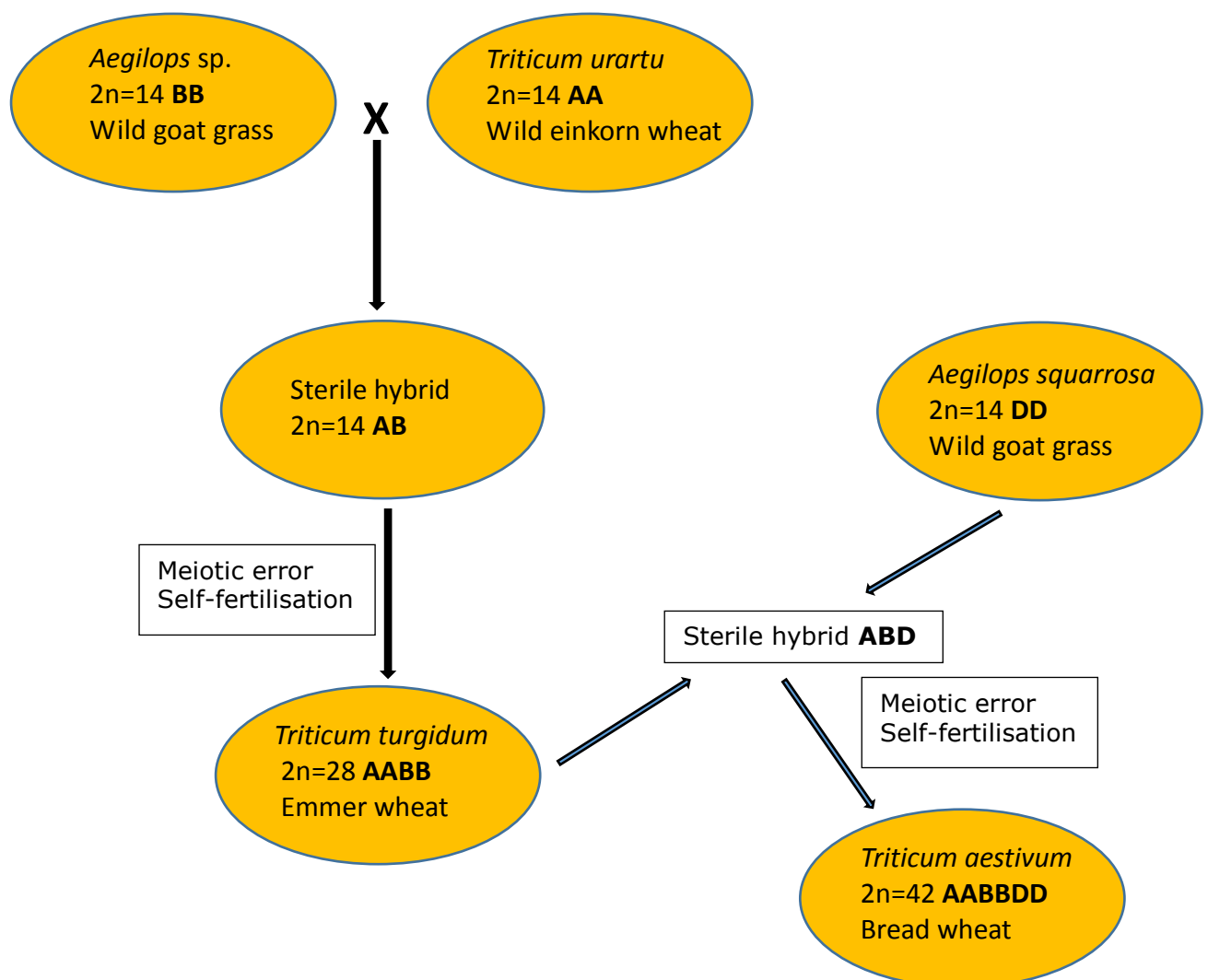
emergence time (reference for each trait given above). The mutation that gave rise to the *Q* allele is the same in tetraploid and hexaploid free-threshing wheat (Simons *et al.*, 2006). The other traits shared by all domesticated wheats are increased seed size, reduced number of tillers, more erect growth and reduced seed dormancy (Feldman, 2001). Grain protein content (GPC) has positive effect on bread and pasta quality, *Gpc-B1* gene was mapped on chromosome arm 6BS (Joppa *et al.*, 1997; Olmos *et al.*, 2003) which showed GPC increase both in tetraploid and hexaploid wheat (Mesfin *et al.*, 1999; Chee *et al.*, 2001). The gene *Gpc-B1* affects seed size and is an early regulator of senescence (high GPC allele lead to shorter duration of grain fill due to early flag leaf senescence) with pleiotropic effects on nitrogen remobilization, total GPC, and grain size. The effect on grain size was more variable depending on the genotype-environment combinations. In some genotypes and environments, the accelerated grain maturity conferred by the functional *Gpc-B1* allele was found to be associated with smaller seeds (Uauy *et al.*, 2006).

### **1.3 Origin of the A, B and D genomes/Evolution**

Allopolyploidy has played a major role in the evolution of crop plants (Zhang *et al.*, 2008). The allopolyploids arose from interspecific hybridisation events followed by spontaneous chromosome doubling (Huang *et al.*, 2002). At the cytogenetic level, bread wheat is an allohexaploid species, having genome composition AABBDD, which arose from two hybridisation events (McFadden and Sears, 1946; Kihara, 1944). The first hybridisation event happened 300,000-500,000 years before present (BP) where the A genome progenitor (*T. urartu*) hybridized with the B genome progenitor (wild goatgrass) to form primitive tetraploid wheat, *T. turgidum* (AABB), where goatgrass contributed the

cytoplasm (female parent) in the hybridization event (Tsunewaki, 1988; Huang *et al.*, 2002). This was followed by the second hybridisation event (9,000 BP) where the cultivated tetraploid, *T. turgidum* (AABB), as female parent, hybridized with the D genome progenitor *Aegilops squarrosa*/*Aegilops tauschii*. Chromosome doubling occurring in this hybrid then resulted in the basic hexaploid configuration (AABBDD) (Kihara, 1944; McFadden and Sears, 1946; Tsunewaki, 1988; Dvorak *et al.*, 1998) (Figure 1.1).

**Figure 1.1:** Evolution of cultivated hexaploid wheat showing two hybridization events



The A and D genomes of allopolyploid wheat share a high degree of homology with the diploid genomes of *T. urartu* and *Ae. tauschii* (Feldman and Levy, 2005). *Ae. tauschii* as the donor of the D genome was confirmed through analysis of DNA sequences of the two genes *Acc-1* (plastid acetyl-coA carboxylase) and *Pgk-1* (plastid 3-phosphoglycerate kinase) and the *GluDy* allele variation (Huang *et al.*, 2002; Giles and Brown, 2006).

It was originally proposed that the B genome donor was based upon *Ae. speltoides* (Tausch) (Sarkar and Stebbins, 1956) which was also supported by Riley *et al.*, (1958) but the specific identity of the B genome donor remains in controversy since then. Feldman. (1979) concluded that *T. longissimum* (*Ae. longissima*) and *T. searsii* (*Ae. searsii*) were candidates for the B genome progenitor. Nath *et al.* (1984) then concluded that *T. searsii* was the possible source of the B genome after studying several likely progenitors with DNA:DNA hybridisations, where *T. turgidum* DNA (labelled with H-thymidine) was hybridized to the unlabelled DNAs of *T. urartu*, *Ae. speltoides*, *T. sharonensis* (*Ae. sharonensis*), *T. bicornis* (*Ae. bicornis*), *T. longissimum*, and *T. searsii*. Dvorak and Zhang. (1990) studies on the distribution of repeated nucleotide sequences among the B-genome chromosomes of *T. aestivum* cv. Chinese Spring showed that the B and G genomes of tetraploid wheats were donated by *Ae. speltoides*. It now seems probable that the B genome donor of wheat was a member of the Sitopsis section of the Triticeae closely related to *Ae. speltoides*, which is either extinct or not yet collected. Another hypothesis proposes that the B genome is of polyphyletic origin, i.e. it is a recombined genome derived from two or more diploid Aegilops species. This is supported by Liu *et al.*, (2003) who showed that a low-copy, non-coding chromosome-specific DNA sequence,



isolated from wheat was mapped to chromosome 3BL. To trace its origin, this sequence was then hybridized to Southern blots containing genomic DNA obtained from cultivars of *T. aestivum*, *T. turgidum*, *T. timopheevii*, *T. urartu*, *T. monococcum*, *Ae. tauschii*, *Ae. speltoides*, *Ae. longissima* and *Ae. sharonensis*.

#### **1.4 Need for use of wild relative species**

During the last 10-20 years in the most wheat grown areas the constant use of the best available cultivars, small initial population size and intense human selection for agronomic traits are thought to have decreased the available genetic diversity in today's cultivated wheat which ultimately affect the yield improvements (Feuillet *et al.*, 2008). This has enhanced the need for new genetic variation. The genetic diversity contained in traditional varieties, crop wild relatives and landraces provide a basis for food production and also act as buffer for adaptation and resilience in the face of the climate change (FAO, 2008b). Hence one possibility is to exploit the genetic variation of the wild relatives of wheat.

#### **1.5 Wild relative introgression/Gene pools**

The gene pool concept by Harlan and de Wet. (1971) provided a useful framework for the utilisation of wheat wild relatives in breeding, where the evolutionary and cytogenetic relationship of wheat with the wild species making up the primary, secondary and tertiary gene pools have been extensively studied (Kihara, 1924, 1944) and methods for gene transfer published (Feldman and Sears, 1981; Jauhar, 1993; Mujeeb-kazi, 1998). The primary gene pool comprises landraces, early domesticated species and wild species that hybridize directly with the cultivated types. Their chromosomes are homologous to the cultivated types and generally undergo homologous recombination following

direct hybridisation and backcrossing, which can thus be exploited in breeding and selection programmes. This gene pool has been most used for crop improvement. The primary gene pool includes the polyploid species, e.g. *T. turgidum* (AABB) and diploid donors of the A and D genomes, *T. urartu* (AA), *T. monococcum* L. (AA), *T. boeoticum* (AA) and *Ae. tauschii* (DD).

The secondary gene pool of wheat contains polyploid species that share at least one homologous genome with cultivated wheat. Gene transfer from these species is possible through homologous recombination when the target gene is located on the homologous genome. This includes species such as *T. timopheevii* (A<sup>t</sup>A<sup>t</sup>GG) and the diploid S-genome (related to the B genome) species from *Aegilops* section *Sitopsis* such as *Ae. speltooides*.

The species lacking A, B, or D genomes belong to the tertiary gene pool. This gene pool contains more distantly related diploids and polyploids. Methods that cause random chromosome breaks or promote homoeologous pairing and recombination have been used in engineering transfers from this gene pool. This group includes most members of the Triticeae that are not within the primary or secondary gene pools e.g., perennials and species from *Secale* spp. (RR), *Thinopyrum* spp. (EE) etc.

Introgressions have been used to improve wheat mainly in terms of disease resistance but also incorporating genes more adapted to changes in environmental conditions. Introgressions have also been used to study the comparative syntenies between wheat and its wild relatives (Qi *et al.*, 2007; King *et al.*, 2017).

### **1.5.1 *Ph1* (pairing homoeologous 1) locus**

The introduction of alien genes into wheat from wild relatives, whose genomes are genetically different from those of wheat, is not easy, as chromosomes of these wild relatives will not normally recombine in wheat/wild relative hybrids without specific strategies being employed. The factors that affect the ability to pair and recombine these chromosomes are often those involved in control of chromosome pairing. In wheat chromosome pairing is confined to only homologous pairs and pairing between homoeologous chromosomes is prevented by the activity of a single locus on the long arm of chromosome 5B (Riley and Chapman, 1958; Riley, 1960; Riley and Kempanna, 1963). This locus preventing homoeologous chromosomes pairing was termed as *Ph1* (pairing homoeologous 1) (Okamoto, 1957) and was localised to a 2.5 Mbp interstitial region of wheat chromosome 5B (Moore, 2002; Griffiths *et al.*, 2006; Al-Kaff *et al.*, 2008). Although the *Ph1* locus was assumed to prevent pairing between homoeologous chromosomes, later with the wheat-rye hybrid chromosome pairing studies Martin *et al.* (2014) suggested that homoeologous pairing is independent of *Ph1*. In wheat, during the telomere bouquet stage the pairing of homologous chromosomes takes place, while the homoeologous pairing starts later during telomere bouquet dispersal. Thus, the *Ph1* locus promotes early homologous pairing and avoids homoeologous pairing in wheat (Martin *et al.*, 2017). In the absence of *Ph1*, homologous pairing is delayed and hence homoeologous pairing take place to a certain extent for example in up to 8 of the 42 chromosomes as reported in Martin *et al.*, 2017.

### **1.5.2 Role of *Ph1* in wheat-wild relative hybrids**

The *Ph1* locus affects the meiotic chromosome pairing in hybrids between wheat and wild relatives. These hybrids possess a haploid complement of each set of chromosomes and therefore only homoeologous chromosomes can pair at meiosis as there are no homologues. In the presence of *Ph1* as there is no recombination occurring in homoeologous chromosomes, the chromosomes are observed as univalents whereas in the absence of *Ph1*, recombination occurs and a number of chromosomes are paired at metaphase I (Dhalinal *et al.*, 1977). Hence, it was desirable to manipulate the *Ph1* locus so that homoeologous pairing and recombination occur between different genomes of wheat and wild relative chromosomes. Sears, (1977) used radiation to produce a *ph1b* mutant stock in Chinese Spring wheat. Since then, lines carrying a deletion of the *Ph1* locus (*ph1b*, *ph1c*) have been used extensively to induce recombination between wheat and wild relative chromosomes, e.g. Sears transferred disease resistance loci from wild relatives (*Agropyron* spp.) of wheat by recombining the chromosomes with those of wheat in lines *Ph1* deficient (Sears, 1972). The transfer of disease resistance genes from *Ae. longissima* using the *ph1b* mutant was also reported (Ceoloni *et al.*, 1988). CIMMYT (International Maize and Wheat Improvement Centre) exploited *ph1* to introgress from closely related species (e.g. *Ae. tauschii*) (William *et al.*, 2005).

### **1.5.3 Introgression strategies**

There are different methodologies for transferring desired traits from wild relative species to wheat, to produce intergeneric and interspecific hybrids.

One of the approaches for wild-relative genome transfer is to cross wheat with the nearest wild relative of interest i.e. addition of one whole wild relative

genome to produce an amphihaploid. The resulting amphihaploids are usually sterile due to genome instability, and therefore to produce fertile hybrids colchicine treatment is used. The colchicine treatment results in the chromosomes of the amphihaploid being doubled and producing a fertile amphidiploid. Spontaneous Triticum x Aegilops hybrids were observed by Leighty and Taylor, (1927), and Vavilov, (1935). Since then, a large number of Triticum x Aegilops hybrids and also reciprocal hybrids have been developed under artificial conditions (Ozkan and Feldman, 2001; Logojan and Molnar-Lang, 2000). Another example of transfer of a whole genome is Triticale, developed after crossing wheat with rye. Triticale is still grown for its combination of grain quality traits (from Triticum) and the vigour and hardiness of *Secale* (Kim *et al.*, 2003). The development of synthetic wheat began in the 1980s when a new synthetic cross was made using a tetraploid wheat and an *Ae. tauschii* accession to increase the genetic diversity in bread wheat (Gill and Raupp, 1987). However, issues such as hybrid necrosis (Pukhalskiy *et al.*, 2000) and excessive hardiness in the rachis and glume have posed problems.

The second approach is the transfer of traits from wild relative species through development of addition, substitution and translocation lines. Due to the presence of the three diverse genomes in wheat and the triplicate nature of the genes, bread wheat tolerates the addition or deletion of pairs of chromosomes, single chromosomes or parts of a chromosome. These lines form the basis of pre-breeding programmes aimed at the transfer of wild relative target traits to cultivated varieties (Feldman and Levy, 2005).

The third approach is production of a recombinant chromosome with desired genes. Certain cytogenetic manipulative approaches collectively termed as

chromosomes engineering were developed by Sears. (1981). These approaches were developed to overcome the linkage drag and reduce size of the wild relative chromosome segment to be transferred into wheat. These cytogenetic manipulative approaches include irradiation, use of gametocidal genes and induced homoeologous pairing (discussed in *Ph1* locus section).

The radiation method allows the transfer of small segments of wild relative by making use of X-rays to induce centromeric breaks and subsequent fusion of wild relative chromatin with that of wheat (Sears and Gustafson, 1993). Sears (1956) used this method to transfer *Lr9*, a leaf rust resistance gene from *Ae. umbellulata* into wheat. This method also facilitated translocation of *Sr26* from *Lophopyrum elongatum* into wheat (Knott, 1987). Gametocidal genes (*Gc*) originated from Aegilops chromosomes (e.g. 4S from *Ae. sharonensis*, Endo, 1990) are groups of selfish genes that induce chromosome breakage in gametes not having them (Tsujimoto, 1995). This mechanism ensures that only gametes containing the *Gc* genes are transmitted. The *Gc* system can be usefully applied in the cases of wild relative chromosomes from species distantly related to wheat showing little tendency to undergo homoeologous recombination with wheat chromosomes. Masoudi-Nejad *et al.* (2002) with the help of *Gc* system transferred wild relative chromosome segments to wheat and rye (Endo *et al.*, 1994; Gyawali *et al.*, 2009, 2010; Li *et al.*, 2013). However both irradiation and use of gametocidal genes give rise to non-compensating chromosomes and hence deleterious effects are often caused as a result of genetic imbalance.

### **1.6 Characterization of wild relative introgressions in wheat genome**

Development of an  $F_1$  hybrid between two distantly related species is not sufficient to conclude successful introgression. If introgression is going to occur

it would be in the gametes of the  $F_1$  hybrid, which is usually very sterile. Therefore introgressions will be found in the first backcross plants generated coming from the  $F_1$  hybrid. Thus, the essential step for introgression is the recombination event(s) which occur in the gametes of the  $F_1$  hybrid.

### **1.6.1 Cytogenetic techniques**

The main conventional techniques used to detect chromosome rearrangement events in the wheat genome are C-banding, genomic *in situ* hybridization (GISH) and fluorescence *in situ* hybridization (FISH). C-banding and N-banding were the first techniques used to identify wild relative chromosomes in the wheat genome (Gerlach, 1977). The more recent microscope-based techniques are GISH and FISH approaches, which can be used to detect the introgressions and translocation breakpoint, effectively complementing classical diagnostic and selection tools for more efficient and precise detection and characterization of the wild relative chromosome segment. GISH based on *in situ* hybridisation with labelled total genomic DNA of the introgressed species on mitotic metaphase preparations of hybrid plants, allows the discrimination of wild relative chromosomes in a wheat background. Further, the Multicolour GISH (mcGISH) technique allows the simultaneous discrimination of several genomes in allopolyploid plants and hence more precise genome analysis. Multicolour GISH using several different genomic probes helps in visualizing two or more genomes in a polyploidy species. For FISH (which has increased the resolution of *in situ* hybridisation) where the hybridisation of repetitive DNA clones reveal the chromosome distribution of these sequences, the availability of suitable probes acts as a backbone, to identify chromosomes and their segments. Examples of probes used in cytogenetic studies of the Triticeae are the pTa71 DNA clone

(Gerlach and Bedbrook, 1979) which identifies the NOR (nucleolus organizing regions) on satellite chromosomes, pAs1 repeat (Rayburn and Gill, 1985) which is used for the identification of D genome chromosomes and the rye subtelomeric repeat pSc119.2 (Bedbrook *et al.*, 1980) used to identify the B-genome chromosomes. The detailed description of the most widely used probe in the Triticeae is as follows:

#### Afa and pSc119.2 sequences in Triticeae

Afa and pSc119.2 are two highly abundant, randomly repeated DNA sequence families in the Triticeae (Rayburn and Gill, 1986a, b; McIntyre *et al.*, 1990). Rayburn and Gill. (1986a, b) first cloned Afa the so called D-genome specific repeat, *Ae. tauschii* as pAs1. Later, Afa was also isolated from a variety of Triticeae members (Nagaki *et al.*, 1995, 1998; Tsujimoto *et al.*, 1997). Nagaki *et al.* (1995) assigned the name "Afa" to a family of the Triticeae repetitive DNA sequences with an average length of ~340 bp and AfaI restriction site (GTAC) at the 150<sup>th</sup> bp position of every monomer. However, the different Triticeae species analyzed, while revealing conserved size of the monomer unit, varied in copy number and restriction sites (Nagaki *et al.*, 1998). Tsujimoto *et al.* (1997) found that the D-genome contains 100-fold more copies of the Afa sequence than the B-genome of wheat. The Afa-family is extensively applied in wheat cytogenetics research, as it produces unique *in situ* hybridization signals on sub-telomeric and interstitial regions of all D-genome chromosomes (Schwarzacher *et al.*, 2011) and can be used to identify recipient wheat chromosomes and wild relative chromatin transfers. pSc119.2, the repetitive DNA sequence of rye origin, is another highly important family of satellite DNA in Triticeae (McIntyre *et al.*, 1990). It is present in the major heterochromatic blocks of wheat and rye



chromosomes and is a family of 120bp repeat units (Taketa *et al.*, 2000; Contento *et al.*, 2005). Bedbrook *et al.* (1980) claimed it to be the first cereal repetitive DNA sequence to be cloned from rye as pSc119. Later, McIntyre *et al.* 1990 sub-cloned pSc119 as pSc119.1, pSc119.2 and pSc119.3 and the sequence analysis revealed only the pSc119.2 contains the 120bp repeat unit sequence. Later, *in situ* and southern hybridization results accompanied with PCR and sequencing showed the presence of these repeat units in different diploid and polyploid Triticeae members (Castilho and Heslop-Harrison, 1995). Similar to the Afa-family, this sequence is also applied in wheat cytogenetic research, as this sequence is part of the large sub-telomeric and intercalary heterochromatic blocks in hexaploid wheat.

Thus, GISH and FISH have been widely used to determine the chromosomal constitution of the wheat/wild relative introgressions in the mitotic metaphase cell stage (Zhan *et al.*, 2015; Danilova *et al.*, 2014). For example, this technique was used in the identification of the 1BL/1RS translocations in wheats (Le *et al.*, 1989). In addition, the combination of the mcGISH technique with sequential FISH on wheat wild relative hybrids enables chromosomes belonging to different genomes to be detected and intergenomic rearrangements involving several genomes to be visualized (Wang *et al.*, 2005; King *et al.*, 2017).

Although all these techniques did help to detect introgressions in wheat/wild relative crossing there were some major drawbacks including limited sensitivity and resolution and the laborious and time consuming nature which limits their use for large scale selection of wheat/wild relative introgressions. Hence to identify small introgressions and chromosome breakpoints in a large population of wheat/wild relative hybrids, quickly and efficiently, there is need of high-

resolution and high-throughput methods of detection, which include the use of molecular markers. So, the GISH and FISH methods can be complemented with exome-captured sequence methods using high-throughput molecular markers (Winfield *et al.*, 2012).

### **1.6.2 Molecular markers**

Depending on the DNA sequence and the nature of the target polymorphism of interest as well as the structure of the genome surrounding the region, a variety of molecular markers could be used to detect polymorphism between different genotypes. The molecular markers can thus be used to identify genetic diversity, allow complex trait dissection, map qualitative and quantitative traits and link these genetic traits to phenotypic variation (Hayward *et al.*, 2015). The first and second molecular marker generations, Restriction Fragment Length Polymorphism (RFLPs) (Van Campenhout *et al.*, 1998), Randomly Amplified Polymorphic DNAs (RAPDs) (Williams *et al.*, 1990), Amplified Fragment Length Polymorphisms (AFLPs) (Vos *et al.*, 1995) and Simple Sequence Repeats (SSR or microsatellite) (Somers *et al.*, 2004) have progressively been used to determine the genetic relationship between individuals in plant breeding. SSR molecular markers have been widely used to tag and map resistance genes in wheat, rye and other wild relatives due to their high level of polymorphism compared with other markers (Somers *et al.*, 2004). However, RFLPs, RAPDs and AFLPs which were the markers of choice during the last two decades, needs gel-based assays and are, therefore, time consuming and expensive. Therefore, emphasis shifted towards the development of molecular markers which can be detected through non-gel-based assays. Although, in wheat, genetic maps were commonly

constructed using SSR markers, the constructed map resolution remains low without a known genome sequence.

Next generation of molecular markers such as single nucleotide polymorphism (SNP) (Akhunov *et al.*, 2009; Allen *et al.*, 2011; Paux *et al.*, 2011; Berkman *et al.*, 2012; Cavanagh *et al.*, 2013), genotype-by-sequencing (GBS) (Edae *et al.*, 2016), diversity array technology (DArT) (Jaccoud *et al.*, 2001; Jing *et al.*, 2009; Kalih *et al.*, 2015; Castillo *et al.*, 2014) and Kompetitive allele-specific PCR (KASP) markers (Rahmatov *et al.*, 2016), specifically used for high-density genetic mapping, have revolutionized the options for marker work. These markers are used in order to precisely locate the monogenic (single gene) or polygenic traits (conferred by more than one gene) of relevance in wheat breeding.

Single nucleotide polymorphisms (SNPs), the most common form of genetic variation among individuals, are the most recently developed DNA marker technology and are considered the preferred marker system for the study of complex genetic traits, such as genome-wide association studies and a wide range of breeding applications including quantitative trait loci mapping, genetic map construction, MAS (marker assisted selection) and population structure analysis (Rafalski, 2002). Their abundance in the genomes of all organisms, amenability to low-cost, high-throughput genotyping and the relatively low mutation rate compared to other markers make SNP markers ideal candidates for studies aimed at the creation of improved cultivars in wheat breeding (Jenkins and Gibson, 2002; Ribaut *et al.*, 2010). The measurement of genetic variation with the help of SNPs starts with the identification or determination of

the genotypes of the particular individuals of the same species, namely “genotyping”.

Recent advances in genotyping technologies have led to an increased use of SNP markers in genetics and genomics (Elshire *et al.*, 2011). Two main platforms exist for high-throughput SNP genotyping : array technologies that incorporate pre-existing sequence knowledge into a closed end assay and open-ended genotyping by sequencing (GBS) that combines genotyping and SNP discovery that can be integrated with methods of exome capture (Chen *et al.*, 2013). These advances have led to an increase in mapping data for wheat (Marone *et al.*, 2012; Cavanagh *et al.*, 2013; Maccaferri *et al.*, 2014, 2015; Wang *et al.*, 2014). The development of SNP-based genotyping platforms has led to an increase in the number of protocols available for analyzing the genetic variation in numerous species (Perkel, 2008). A number of platforms have recently been developed to perform high-density genotyping and these have been successfully employed to genotype wheat (Chao *et al.*, 2010). High-throughput sequencing technologies including chips of 9000 SNPs (9K SNP chip genotyping) and 90,000 SNPs (90K SNP chip genotyping), DArT-Seq and GBS (~41000 SNPs) has also been developed for the use in wheat (Allen *et al.*, 2011; Poland *et al.*, 2012; Li *et al.*, 2015). Multiplexed shotgun genotyping which is a low coverage genotyping approach, have been made available (Davey *et al.*, 2011). To detect the smallest translocation/introgression breakpoints possible in the wheat relatives and to produce small wild relative transfers without linkage drag in wheat, high-density molecular markers such as SNP and GBS are widely used. Tiwari *et al.* (2014) with the help of genome-specific SNP markers validated wheat lines containing the *Yr40/Lr57* resistance genes from *Ae. geniculata* which

showed reduced linkage drag compared to the previous mapping done using RFLP analysis which did not detect the small chromosome segment (Kuraparthi *et al.*, 2007). Gao *et al.* (2015) used GBS and array derived SNP markers to map the stem rust resistance gene *Sr42* on chromosome arm 6DS, which were then converted to KASP assays. The 9K SNP wheat chip was used to detect genomic regions targeted by breeding and improvement selection in wheat (Cavanagh *et al.*, 2013). Whole genome shotgun sequencing resulted in the construction of a chromosome-based draft sequence of hexaploid wheat (Brenchley *et al.*, 2012; Consortium 2014).

#### Molecular maps in wheat

Marker assisted breeding enhances the exploitation of wild relatives of crops for useful genetic variation (Tanksley and McCouch, 1997). Molecular markers in bread wheat have been used for the preparation of genetic and physical maps.

#### Genetic Maps:

Genetic maps are based on the recombination frequency between markers, measured in centimorgans (cM). In order to create genetic maps the isolation of informative DNA markers is required. Both qualitative and quantitative traits controlled by a number of genes have already been tagged or mapped using a variety of molecular markers (McIntosh *et al.*, 2008). The first DNA marker system used to construct the linkage maps of wheat in a recombinant inbred population, were the RFLPs (Restriction fragment length polymorphism) (van Deynze *et al.*, 1995; Marino *et al.*, 1996). Later, SSR maps of wheat, such as for hexaploid wheat were constructed, leading to improved density of wheat genetic maps (Somer *et al.*, 2004; Song *et al.*, 2005).

### Physical Maps

Physical maps are based on the number of nucleotide pairs, measured in kilobases (Kb). The physical maps are thus compared to the genetic map to identify and clone genes and also reveal variation in recombination frequencies (Gupta *et al.*, 2008). Thus, the physical maps helps in the identification of genes and the genetic maps helps in linking the genes to the target trait under study. Using the deletion stocks, genes for morphological characters were also mapped to physical segments of all wheat chromosomes with help of RFLP, isozyme, biochemical markers, seed protein data and ISH (*in situ* hybridisation) (Endo *et al.*, 1991; Mukai and Endo, 1992; Yamamori *et al.*, 1994; Delaney *et al.*, 1995a,b; Endo and Gill, 1996). Qi *et al.* (2004) found that chromosome bin map of ESTs is a unique resource for comparative mapping and structural and functional analysis of the wheat genome and successfully mapped 16,000 EST loci and 725 SSR loci across the wheat genome.

### Comparative Genetic Maps

Comparative mapping involves the alignment of chromosomes of related species based on the genetic mapping of common molecular markers. The comparison of the genomes of two related species can help locate important genes that have been identified in one species but not in another together with their evolutionary relationship. As the relationships between an increasing number of species were unraveled, a new visualization method that could display multiple comparisons in an adequate way, the first 'crop circle' diagram was published (Moore *et al.*, 1995). Gale *et al.* (2002) made available a Consensus map of 12 grass genomes including wheat representing chromosomes segments of each genome relative to those in rice based on the mapping of anchor molecular markers. Numerous

genes using comparative genomics were discovered such as the pairing gene *Ph1* when wheat ESTs were compared with *Brachypodium distachyon* (Huo *et al.*, 2006), the *Phs* gene controlling preharvest sprouting (Gale *et al.*, 2002), the *Ha* gene for grain hardness (Chantret *et al.*, 2004), the *Rg* and *Bg* genes for red and black glume coloration (Khlestkina *et al.*, 2006) and the *Pm3* gene for resistance against powdery mildew (Wicker *et al.*, 2007).

### **1.7 Success of wild relative gene transfers:**

In the past years, the wild relatives have been exploited for transferring agronomically desirable genes such as disease resistance to wheat with the introgression strategies discussed above. Some examples of wild relative genes that have been transferred successfully in wheat in the past are given in Table 1.2.

**Table 1.2:** Different wild relatives gene introgression into wheat conferring resistance to a range of diseases and other agronomically important traits.

| Source                | Trait                           | Gene/allele/<br>QTL                      | Location    | Reference                       | Remarks                                                                                                                                            |
|-----------------------|---------------------------------|------------------------------------------|-------------|---------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------|
| <i>Secale cereale</i> | Powdery mildew resistance       | <i>Pm7</i>                               | 4BS 4BL-2RL | Friebe <i>et al.</i> , (1996)   | Increased biological yield by 11-12%, drought tolerance, higher biomass, better phosphorus extraction; Carver and Rayburn, (1994); Rajaram, (2001) |
|                       | Yellow rust resistance          | <i>Pm20</i>                              | 6BS-6RL     |                                 |                                                                                                                                                    |
|                       |                                 | <i>Pm8</i>                               | 1RS-1BL     | Hsam and Zeller, (1997)         |                                                                                                                                                    |
|                       |                                 | <i>Yr9</i>                               | 1B.1RS      | Mago <i>et al.</i> , (2002)     |                                                                                                                                                    |
| <i>Ae. tauschii</i>   | Powdery mildew resistance       | <i>Pm2</i>                               | 5DS         | Lutz <i>et al.</i> , (1995)     |                                                                                                                                                    |
|                       |                                 | <i>Pm19</i>                              | 7D          | Lutz <i>et al.</i> , (1995)     |                                                                                                                                                    |
|                       |                                 | <i>Pm34</i>                              | 5DL         | Miranda <i>et al.</i> , (2006)  |                                                                                                                                                    |
|                       |                                 | <i>Pm35</i>                              | 5DL         | Miranda <i>et al.</i> , (2007)  |                                                                                                                                                    |
|                       |                                 | Leaf rust resistance                     | <i>Lr40</i> | 1DS                             |                                                                                                                                                    |
|                       | Yellow rust resistance          | <i>Yr28</i>                              | 4DS         | Singh <i>et al.</i> , (2000)    |                                                                                                                                                    |
| <i>T. monococcum</i>  | Powdery mildew resistance       | <i>Pm4</i>                               | 2AL         | Schmolke <i>et al.</i> , (2012) | Karnal bunt resistance (Vasu <i>et al.</i> , 2000)                                                                                                 |
|                       | Cereal cyst nematode resistance | <i>Qcre.pau-1A</i><br><i>Qcre.pau-2A</i> | 1A<br>2A    | Singh <i>et al.</i> , (2010)    |                                                                                                                                                    |
|                       | Stripe rust resistance          | <i>QYrtm.pau-2A</i>                      | 2A          | Chhuneja <i>et al.</i> , (2008) |                                                                                                                                                    |
| <i>T. boeoticum</i>   | Fe micronutrient                | <i>Qfe.pau-2A</i><br><i>Qfe.pau-7A</i>   | 2A<br>7A    | Tiwari <i>et al.</i> , (2009)   |                                                                                                                                                    |
|                       | Zn micronutrient                | <i>Qzn.pau-7A</i>                        | 7A          | Tiwari <i>et al.</i> , (2009)   |                                                                                                                                                    |
|                       | Powdery mildew resistance       | <i>Pmtb7A.1</i> and <i>Pmtb7A.2</i>      | 7A          | Chhuneja <i>et al.</i> , (2012) |                                                                                                                                                    |
| <i>Ae. speltoides</i> | Powdery mildew resistance       | <i>Pm32</i>                              | 1BL         | Hsam <i>et al.</i> , (2003)     |                                                                                                                                                    |
|                       |                                 | <i>Pm12</i>                              | 6BS-6SS-6SL | Jia <i>et al.</i> , (1996)      |                                                                                                                                                    |
|                       | Stem rust resistance            | <i>Sr39</i>                              | 2BS         | Mago <i>et al.</i> , (2009)     |                                                                                                                                                    |



|                          |                                 |                   |                 |                                                           |                                                                                                                                                     |
|--------------------------|---------------------------------|-------------------|-----------------|-----------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------|
|                          |                                 | <i>Sr54</i>       | 2D              | Ghazvini <i>et al.</i> , (2013)                           |                                                                                                                                                     |
|                          | Leaf rust resistance            | <i>Lr28</i>       | 4AL             | Naik <i>et al.</i> , (1998)                               |                                                                                                                                                     |
| <i>Ae. longissima</i>    | Powdery mildew resistance       | <i>Pm13</i>       | 3BL-3SS-3S      | Ceoloni <i>et al.</i> , (1992)                            |                                                                                                                                                     |
| <i>Haynaldiavillosa</i>  | Powdery mildew resistance       | <i>Pm21</i>       | 6VS-6AL         | Xie <i>et al.</i> , (2012)<br>Chen <i>et al.</i> , (2013) |                                                                                                                                                     |
| <i>Dasypyrumvillosum</i> | Stem rust resistance            | <i>Sr52</i>       | T6AS.6V#3L      | Qi <i>et al.</i> , (2011)                                 | V genome genes for elevated seed protein content and positive effect on gluten strength (Shewry <i>et al.</i> , 1991)                               |
|                          | Powdery mildew resistance       |                   |                 | Cao <i>et al.</i> , (2011)                                |                                                                                                                                                     |
| <i>T. dicoccoides</i>    | Powdery mildew resistance       | <i>Pm26</i>       | 2BS             | Rong <i>et al.</i> , (2000)                               | Gene for tan spot located on 5B and 3B (Singh <i>et al.</i> , 2006)<br>Increased protein content and protein quality traits (Sharma and Gill, 1983) |
|                          |                                 | <i>Pm30</i>       | 5BS             | Liu <i>et al.</i> , (2000)                                |                                                                                                                                                     |
|                          |                                 | <i>Pm36</i>       | 5BL             | Blanco <i>et al.</i> , (2008)                             |                                                                                                                                                     |
|                          |                                 | <i>Pm31(MIG)</i>  | 6AL             | Xie <i>et al.</i> , (2003)                                |                                                                                                                                                     |
|                          |                                 | <i>Pm41</i>       | 3BL             | Li <i>et al.</i> , (2009)                                 |                                                                                                                                                     |
|                          |                                 | <i>mliW72</i>     | 7AL             | Ji <i>et al.</i> , (2008)                                 |                                                                                                                                                     |
|                          | Yellow rust resistance          | <i>Yr35</i>       | 6BS             | Dadkhodaie <i>et al.</i> , (2011)                         |                                                                                                                                                     |
| <i>A. ovata</i>          | Powdery mildew resistance       | <i>Pm29</i>       | 7DL             | Zeller <i>et al.</i> , (2002)                             |                                                                                                                                                     |
| <i>Ae. geniculata</i>    | Powdery mildew resistance       | <i>Pm29</i>       | 7D              | Stoilova and Spetsov, (2006)                              |                                                                                                                                                     |
|                          | Leaf and yellow rust resistance | <i>Lr57, Yr40</i> | <i>T5DL-5MS</i> | Kuraparthi <i>et al.</i> , (2007)                         |                                                                                                                                                     |
| <i>T. carthlicum</i>     | Powdery mildew resistance       | <i>Pm33</i>       | 2BL             | Zhu <i>et al.</i> , (2005)                                |                                                                                                                                                     |
| <i>Th. intermedium</i>   | Powdery mildew resistance       | <i>Pm43</i>       | 2DL             | He <i>et al.</i> , (2009)                                 | Improved seed storage protein quality for 1D (Cao <i>et al.</i> , 2007)                                                                             |
|                          | Stem rust resistance            | <i>Sr43</i>       | 7D              | Kim <i>et al.</i> , (1993)                                |                                                                                                                                                     |
|                          | Leaf rust resistance            | <i>Lr38</i>       | 1DS-1DL         | Friebe <i>et al.</i> , (1996)                             |                                                                                                                                                     |
|                          | Yellow rust resistance          | <i>Yr50</i>       | 4BL             | Liu <i>et al.</i> , (2013)                                |                                                                                                                                                     |

|                                              |                                                               |                   |            |                                   |                                                                                                                              |
|----------------------------------------------|---------------------------------------------------------------|-------------------|------------|-----------------------------------|------------------------------------------------------------------------------------------------------------------------------|
| <i>T. turgidum</i>                           | Powdery mildew resistance                                     | <i>PmG25</i>      | 5BL        | Alam <i>et al.</i> , (2013)       |                                                                                                                              |
|                                              | Yellow rust resistance                                        | <i>Yr26</i>       | 1BS        | Ma <i>et al.</i> , (2001)         |                                                                                                                              |
| <i>Th. ponticum</i>                          | Stem rust resistance                                          | <i>Sr26</i>       | T6A-6Ae#1L | Friebe <i>et al.</i> , (1996)     | Group 7 chromosomes of wheat/ <i>Th. ponticum</i> carry genes for yield-contributing trait (Kuzmanovic <i>et al.</i> , 2014) |
| <i>Th. Ponticum</i> and <i>Th. distichum</i> | Leaf rust resistance                                          | <i>Lr19</i>       | 7DL        | Prins <i>et al.</i> , (1996)      |                                                                                                                              |
| <i>Ae. comosa</i>                            | Stem rust resistance                                          | <i>Sr34</i>       | 2A         | Xu <i>et al.</i> , (2008)         |                                                                                                                              |
| <i>Ae. searsii</i>                           | Stem rust resistance                                          | <i>Sr51</i>       | 3AL        | Liu <i>et al.</i> , (2011)        |                                                                                                                              |
| <i>Ae. cylindrica</i>                        | Leaf rust resistance                                          | <i>Lr39, Lr41</i> | 2DS        | Singh <i>et al.</i> , (2003)      |                                                                                                                              |
| <i>Ae. kotschy</i>                           | Leaf rust resistance                                          | <i>Lr54</i>       | 1D         | Marais <i>et al.</i> , (2005)     |                                                                                                                              |
|                                              | Yellow rust resistance                                        | <i>Yr37</i>       | 2D         | Marais <i>et al.</i> , (2005)     |                                                                                                                              |
| <i>Ae. sharonensis</i>                       | Leaf rust resistance                                          | <i>Lr56</i>       | 6A         | Marais <i>et al.</i> , (2006)     |                                                                                                                              |
|                                              | Yellow rust resistance                                        | <i>Yr38</i>       | 6A         | Marais <i>et al.</i> , (2010)     |                                                                                                                              |
| <i>Ae. triuncialis</i>                       | Leaf rust resistance                                          | <i>Lr58</i>       | 2BL        | Kuraparthi <i>et al.</i> , (2007) |                                                                                                                              |
| <i>T. spelta</i>                             | Yellow rust resistance                                        | <i>Yr5</i>        | 2BL        | Singh <i>et al.</i> , (2007)      | Two genes for Septoria resistance (Simon <i>et al.</i> , 2005, 2010)                                                         |
| <i>Ae. neglecta</i>                          | Yellow rust resistance                                        | <i>Yr42</i>       | 6AL(6L.6S) | Marais <i>et al.</i> , (2009)     |                                                                                                                              |
| <i>T. durum</i>                              | Yellow rust resistance                                        | <i>Yr53</i>       | 2BL        | Xu <i>et al.</i> , (2013)         |                                                                                                                              |
| <i>Ae. caudata</i>                           | Leaf rust resistance                                          | <i>LrAc</i>       |            | Riar <i>et al.</i> , (2012)       |                                                                                                                              |
| <i>Ae. umbellulata</i>                       | Leaf rust resistance                                          | <i>LrU1, LrU2</i> |            | Chuuneja <i>et al.</i> , (2008)   |                                                                                                                              |
|                                              | Yellow rust resistance                                        | <i>YrU1</i>       |            | Chuuneja <i>et al.</i> , (2008)   |                                                                                                                              |
| <i>Ag. elongatum</i>                         | Improved water stress adaption, higher root and shoot biomass |                   |            | Placido <i>et al.</i> , (2013)    | Blue aleurone feature, hence source of beneficial flavonoids (Shen <i>et al.</i> , 2013)                                     |
| <i>Th. junceum</i> and                       | FHB resistance, tan spot,                                     |                   |            | McArthur <i>et al.</i> , (2012)   |                                                                                                                              |

|                          |                                                                                           |  |  |                                                                                                                                 |  |
|--------------------------|-------------------------------------------------------------------------------------------|--|--|---------------------------------------------------------------------------------------------------------------------------------|--|
| <i>Elymus rectisetus</i> | Stagonosporanodorum blotch, stem rust                                                     |  |  |                                                                                                                                 |  |
| <i>Leymus racemosus</i>  | FHB, salt tolerance, Aluminium tolerance, biological nitrification inhibition             |  |  | Chen <i>et al.</i> , (2005)<br>Liu <i>et al.</i> , (2001)<br>Mohammed <i>et al.</i> , (2013)<br>Subbarao <i>et al.</i> , (2007) |  |
| <i>Ae. biuncialis</i>    | Quality-associated HMW glutenin subunits, Powdery mildew resistance, Stem rust resistance |  |  | Zhou <i>et al.</i> , (2014)                                                                                                     |  |

## 1.8 Negative effects related to wild relative genes

The successful transfer of a chromosome segment from wild relative species frequently comes with some disadvantages which prevent the achievement of the desired benefits from them. Introgressions in addition to useful genes often contain genetic material that can negatively influence the expression of agronomically valuable traits. The *Lr9* gene from *Ae. umbellulata* considerably decreased the yield in wheat lines (Friebe *et al.*, 1996). The *Lr19* gene from *Ag. elongatum*, is linked with the *Y* gene which increases the amount of yellow pigment in endosperm (Knott, 1968; Uhrin *et al.*, 2008) and hence its use in wheat varieties is limited in those countries that give preference to white flour, although its presence has been found to have a positive effect on yield (Singh *et al.*, 1998). *Lr38*, a leaf rust resistant gene seems to result in a considerable decrease in grain yield (Dych and Friebe, 1993). *Lr47*, a gene from *Ae. speltoides* has a negative influence on agronomic and qualitative traits (Brevis *et al.*, 2008) and Kumlay *et al.* (2003) indicated that the presence of the wheat-rye 1B.1R translocation, which contains the *Lr26*, *Pm8*, and *Sr31* genes, often results in a decrease in the quality of the flour and bread.

Linkage drag is thus a real challenge for wheat breeders carrying out wild relative introgression. One example where the linkage drag has been overcome is the resistance gene against eyespot (*Pch1*), introduced from *Ae. ventricosa* which had a closely linked gene affecting yield performances. After a selection programme this unfavourable linkage was broken and cultivars with the *Pch1* gene such as Lynx were developed which exhibited no yield losses (Worland and Snape, 2001). The negative influence of wild relative material can be associated with the pleiotropic effects of the resistance genes themselves i.e. sometimes

introgressed wild relative genes affect more than one trait and the introgressions are only useful if such effects are positively associated with desirable traits. For example, the *mlo* locus which is responsible for resistance to powdery mildew in barley causes a decrease in the grain yield by 4% (Kjaer *et al.*, 1990).

The major negative effect using radiation for wild relative transfer is that the radiation causes random breakage, which thus leads to a non-compensating introgression or translocation. Hence, important wheat genes may be lost as they are not replaced with the copies from the wild relative. Therefore, introgressions introduced via homoeologous recombination are considered to be the most effective.

### **1.9 Aims and objectives of the study:**

The main aims of the study are:

- 1) Use of a whole-genome introgression approach; the attempt to transfer chromosome segments from the entire genome of *T. timopheevii* into hexaploid wheat (cv. Paragon).
- 2) Detect and characterize the introgressions via a wheat/wild relative SNP array (Axiom 35K genotyping array)
- 3) Validation of the genotyping with the use of fluorescence *in situ* hybridisation in some introgression lines where whole chromosome have been transferred.
- 4) Development of KASP markers to track introgressions in different generations.
- 5) Phenotyping of wild *T. timopheevii* accessions for target traits such as mineral analysis and Fusarium head blight (FHB).

## **Chapter 2**

### **Materials and methods**

#### **2.1 Plant Material**

Introgressions of *T. timopheevii* into hexaploid wheat were produced by crossing the *T. timopheevii* as the male parent with hexaploid wheat as the female parent. F<sub>1</sub> plants were backcrossed to hexaploid wheat and backcrossing was continued to isolate genotypes containing individual introgressions before self-pollination to produce homozygous introgressions. The crossing scheme followed is shown in Chapter 3, section 3.2.2.

#### **2.2 Seed sterilisation and transfer to soil**

Seeds were sterilised with 1ml 5% ClNaO (10% solution-sigma Aldrich, Appendix 1), followed by shaking for 15 minutes. The seeds were then transferred to fresh sterile water for 2 days at 4° C to soften the seed before embryo dissection.

Germinated seeds were sown directly into soil (John Innes compost No. 2) in moduled trays, whilst seeds which did not show germination were then embryo rescued and placed on culture medium (MS + 2% sucrose + 1% agarose, Appendix 1).

#### **2.3 Vernalisation**

Germination dates were recorded and the moduled trays were transferred to vernalisation. Spring wheat seedlings were vernalized at 6°C for 4 weeks, wild accessions for 8 weeks and backcrosses from 4 to 8 weeks depending on the generation of backcross.

At the end of the vernalisation treatment the trays were placed in the glasshouse (16 hours light at 20°C and 8 hours dark at 15°C) and the plants were potted individually in 2 litres pots.

## **2.4 DNA extraction**

One to two pieces of young leaf (3-4 cm) were harvested into a 2ml tube (~1cm) and the tubes frozen in liquid nitrogen. The leaves were then broken into small pieces with a cold pipette tip and transferred to a cold 2ml tube. One 5mm stainless steel ball was added to each tube.

The samples were ground using a Qiagen Retsch mill, (Type MM 300) for 6.5 mins at a frequency of 20 Hz/second. 800µl of extraction buffer (Appendix 1) was added and the tubes shaken thoroughly before being incubated at 65°C for 1 hour. After incubation, the tubes were transferred to a fridge at 4°C for 20 mins to cool down to room temperature. 400µl of 6M ammonium acetate (Appendix 1) (at 4°C) was then added and the tubes again shaken vigorously to mix well and left for 15-30 minutes in the fridge. The tubes were centrifuged for 10 mins at 13000 rpm (11952 g). The supernatant was then transferred to a new 2ml tube and 400µl of iso-propanol was added to each sample.

The samples were mixed thoroughly and the DNA was then allowed to precipitate for 10-15 minutes in the fridge (4°C). The samples were centrifuged for 10 minutes at 10000 rpm (11963 g) to pellet the DNA and the supernatant removed. The pellet was washed in 400µl 70% ethanol and vortexed briefly. The tubes were then centrifuged for 3 minutes at 13000 rpm and the supernatant again tipped off. The tubes containing the pellet were incubated at 37°C for 5-10 minutes to dry the pellet.

The pellet was re-suspended in 100-200 µl of dH<sub>2</sub>O. The DNA was mixed by gently tapping the tube and then left overnight at 4°C. The following day the tubes were centrifuged for 5 minutes at 13000 rpm and the supernatant transferred to a fresh tube. The DNA was stored at -20°C.

The DNA extracted for Genomic *in situ* hybridisation and fluorescent *in situ* hybridisation had an additional phenol chloroform step. 300ul of phenol/chloroform (1:1 V/V) was added before the 70% ethanol wash step. The samples were mixed gently and then centrifuged for 5 mins at 13000 rpm. The supernatant was then transferred to clean tubes and the ethanol wash step carried out as above.

### **Assessment of quality and quantity of DNA**

The quantity of DNA was checked using either a Qubit 2.0 fluorometer (Invitrogen™) or a Nanodrop 2000 spectrophotometer (Thermo Scientific), and was estimated in ng/µl. Dilutions were then made using the numerical formula  $N_1 \times V_1 = N_2 \times V_2$ , where N is the normality and V is the volume and 1 and 2 are the initial and final values.

For example: if the DNA concentration with Qubit for a sample is 600 ng/µl and the final concentration needed is 50 ng/µl

$$\begin{aligned}N_1 \times V_1 &= N_2 \times V_2 \\600 \times V_1 &= 50 \times 50 \\V_1 &= 2500/600 \\&= 4.16 \mu\text{l}\end{aligned}$$

Therefore the required volume is 4.16 µl of DNA + 45.84 µl water



## **2.5 Genomic *in situ* hybridisation (GISH) and Fluorescence *in situ***

### **hybridisation (FISH):**

#### **2.5.1 Root tips and nitrous oxide treatment**

Seeds were germinated on damp Wattman filter paper in petri-plates in the dark for 2 days. When the roots were 2-3cm long, 1-2 roots were cut off and placed in a 0.5ml tube (dampened by spraying a thin layer of dH<sub>2</sub>O into the tubes) and the tubes placed on ice. The tubes were then transferred to a solid steel chamber filled with nitrous oxide at 10 bar (atmospheric pressure) for 2 hrs. The roots were then fixed in 90% acetic acid for 10 mins before washing three times with dH<sub>2</sub>O. The roots were transferred to new tubes and stored in 70% ethanol at -20°C. The seedlings from which the roots were sterilised and potted out as in sections 2.2 and 2.3 above

#### **2.5.2 Making Probes**

Nick translation is a technique for radioactively labelling double-stranded DNA, making it suitable as a hybridization probe for detecting specific genomic sequences (Rigby *et al.*, 1997). Nick translation introduces random 'nicks' in DNA. DNA polymerase I adds the fluorophore-labeled nucleotides to the 3' end terminus while simultaneously eliminating the pre-existing unmodified nucleotides from the 5' end terminus of the nicks.

### 2.5.2.1 Nick translation reaction 20 µl:

**Table 2.1** Nick translation reaction components and their volume in micro litre (µl) used to make probes.

| Components                           | Volume (µl) |
|--------------------------------------|-------------|
| ddH <sub>2</sub> O                   | 9.85        |
| DNA                                  | 3           |
| 10x Nick translation buffer          | 2           |
| Non-labelled dNTPs (2mM each, mixed) | 2           |
| Labelled dNTP (1mM)                  | 0.5         |
| DNA Polymerase I (10 U/µl)           | 5           |
| DNase (100 mU/µl) diluted            | 0.8         |

The solution was mixed by pipetting and incubated at 16°C for 2 hours in the dark.

### 2.5.2.2 Ethanol precipitation of the probe

160µl of SS DNA (single stranded DNA) (Appendix 1) working solution was added to the nick translation reaction and vortexed. Further 25x of the reaction volume of 9:1 (V/V) 100% ethanol: 3M sodium acetate solution (Appendix 1) (e.g. for 500 µl- 450 µl 100% ethanol + 50µl 3M sodium acetate solution) was added to the reaction mixture before being incubated at -20°C overnight.

The reaction mixture was centrifuged at 12,000 rpm for 30 minutes at 4°C and the supernatant discarded. The probe pellet was washed with 70% ethanol and centrifuged for 5 mins (at 12,000 rpm) twice. The probe was air-dried in the dark for 5-10 mins. The pellet was then dissolved in 20 µl of 2x SSC + 1xTE buffer (Appendix 1) and stored at -20°C in the dark.

### **2.5.3 Preparation of blocking genomic DNA**

500–700 µl genomic DNA being used as blocking DNA was then aliquoted into a 1.5 ml tube. The eppendorf tube cap was then sealed tightly with NASCO film and the DNA was treated in a pressure cooker at 115°C for 8 mins.

### **2.5.4 Preparation of enzyme solution**

10 g of the enzyme solution was made on ice by mixing the enzyme and buffer in the concentration shown below:

|                                       |       |
|---------------------------------------|-------|
| Pectolyase Y-23 (1% w/w)              | 0.1g  |
| Cellulase Onozuka R-10 (2% w/w)       | 0.2g  |
| 1X Citric Buffer (pH5.5) (Appendix 1) | 9.7 g |

The enzyme solution was divided into 15–20 µl aliquots and stored at -20°C.

### **2.5.5 Preparation of metaphase spreads from root tips**

Metaphase spreads were prepared from root tips using a nitrous oxide-enzymatic maceration method (Kato, 1999).

The roots stored in ethanol at -20°C (section 2.3.1) were washed with dH<sub>2</sub>O three times for 10 mins per wash. The roots were then put on filter paper to remove excess dH<sub>2</sub>O. The actively dividing region of the root tip (~ 1mm) in length was cut out and transferred to a 0.5ml tube containing 20 µl enzyme solution (Pectolyase 1% and Cellulase 2%, see section 2.5.4).

The tubes were incubated at 37°C for 45 min for *T. timopheevii* accession (P95-99.1-1), 46 min for Paragon and 48 mins for all the *T. timopheevii* backcrosses. After incubation, the tubes were put on the ice immediately and the roots washed three times with 150 µl of 70% ethanol. The root tips were stored in 100ul of 70% ethanol at -20°C until required.

In order to make slides, a suspension of the metaphase cells was prepared. Each root tip was transferred to a clean tube and a small drop of 70% ethanol added. The root tips were then pressed against the tube wall with a dissection needle to make the cell suspension. The tubes were then tapped gently several times to suspend the cells.

The tubes were centrifuged at 5000 rpm for 1.5 mins. The supernatant was removed carefully from the tube without disturbing the cell pellet. The cell pellet was dried briefly by keeping the tube lid open for 6 minutes. 35 µl of 100% acetic acid was then added to the tubes and the tubes were kept on ice for 1 hr 45 minutes. Glass slides (Thermo Scientific D-38116, 76 x 26 mm) were placed on plastic rods in a moist chamber (a cardboard box with several layers of wet tissue over a water proof film). The tubes tapped gently and the solution mixed with a micropipette tip. 7µl of cell suspension was then dropped on to each slide from a height of 5-10 cm above the slide. The cardboard box was then closed and the slides left to gradually dry out at room temperature for approximately 10 mins. Slides selected for GISH were treated twice with UV light at 0.125 Joules to cross link the chromatin on the slides. The remaining slides were stored in a light proof box at -20°C.

#### **2.5.6 GISH (Genomic *in situ* hybridisation)**

The GISH probe mixture was made up of the following:

|                        |                           |
|------------------------|---------------------------|
| Probe gDNA (100ng/µl)  | - 2µl/slide               |
| 40x Blocking DNA       | - 5µl/slide               |
| Hybridisation solution | - 20µl/slide (Appendix 1) |

#### Probe Denaturation:

The probe mixture was denatured in a PCR machine (Eppendorf Master-cycler nexus GSx1, Applied Bio systems Gene Amp 9700) at 85°C for 8 minutes and then immediately placed on ice.

The probe mix (22 µl) was added to the cover slip (24 x 32 mm Plastic, VWR International Ltd) and the cover slip was thus put on the slides which were cross linked previously.

#### Denaturation of slides:

The slides were then denatured in a PCR machine at 75°C for 5 mins 30 secs.

#### Hybridisation of slides:

The slides were placed on a slide holder in a moist chamber (see section 2.5.6 above) and incubated at 42°C overnight.

#### Post hybridisation washing:

The slides were dipped into 2x SSC (Appendix 1) twice in a water bath at 37°C, to make the cover slip fall off the slides. The second wash was done with 4x SSC with added tween (Appendix 1) in the water bath at 37°C for 3 mins and excess SSC was absorbed from the side of the slides with the help of tissue paper. 12 µl of Vectashield® (H-1200, Vector Laboratories, Inc. Burlingame, CA 94010) mounting medium with DAPI (Appendix 1) [or 1:2 diluted with 1x PBS (Phosphate-buffered saline) (Appendix 1)] was added to new cover slips and these cover slips were then put onto the slides avoiding air bubbles. The slides were then observed with a multi-filter microscope (Leica Microscope DM1000 LED, ZEISS Metasystems MF-1377).

### 2.5.7 FISH (Fluorescence *in situ* hybridisation)

Two oligonucleotide probes, pSc119.2 and Afa family were used for the fluorescence *in situ* hybridisation. pSc119.2 (a rye subtelomeric heterochromatic sequence – Bedbrook *et al.*, 1980, was labelled with Alexa Fluor-488 (green) while the Afa family [a subfamily clone of the pAs1 repetitive sequence originally cloned by Raybun and Gill (1986a, b) from *Aegilops squarrosa*] was labelled with Alexa Fluor-594 (red). The PCR sequence of the probes was directly labelled with nick translation.

The primer sequence of both the oligonucleotide probes is given in Table 2.2 below:

**Table 2.2:** Primer sequence of oligonucleotide probes used for FISH

| Serial number | Primers | Sequence                   |
|---------------|---------|----------------------------|
| 1             | pScF25  | 5'-GTGCTGATGACCGAAACG-3'   |
|               | pScR147 | 5'-GCACTCGCAGTTTTGGCCG-3'  |
| 2             | AS-AF   | 5'-GATGATGTGGCTTTGAATGG-3' |
|               | AS-BR   | 5'-GCATTTCAAATGAACTCTGA-3' |

#### 2.5.7.1 PCR amplification

Amplification of the probes was carried out using PCR in 0.5 ml Eppendorf tubes in an Eppendorf Master Cycler (Eppendorf Master-cycler nexus GSx1, Applied Biosystems Gene Amp 9700) using Rye DNA (concentration-50 ng/μl) as a template for pSc119.2 and Chinese spring DNA (concentration-50 ng/μl) as a template for Afa-family in a final volume of 25 μl per reaction. The stock and final concentrations is given in Table 2.3.

**Table 2.3:** Final Concentrations of different components for PCR reaction used for amplification of FISH probes.

| Components                                       | Volume (μl) |
|--------------------------------------------------|-------------|
| Genomic DNA(~50ng/μl)                            | 2           |
| ddH <sub>2</sub> O                               | 17.8        |
| 10x Buffer with Mg++                             | 2.5         |
| dNTP's (10μm each)                               | 0.5         |
| Primer Mix (pm) (Forward and reverse,10 μm each) | 2           |
| Taq polymerase                                   | 0.2         |
| Total                                            | 25          |

The reaction mixture was placed in a 96 well thermal cycler. Amplification was performed using the temperature profiles shown below in Table 2.4 and Table 2.5.

**Table 2.4:** Temperature profile used for PCR of pSc119.2 probe

| Step                 | Temperature(°C) | Time (minutes) | No. of cycles |
|----------------------|-----------------|----------------|---------------|
| Initial denaturation | 94.0            | 03:00          |               |
| Denaturation         | 94.0            | 00:30          | x6            |
| Annealing            | 65.0*           | 00:30          |               |
| Elongation           | 72.0            | 00:45          |               |
|                      | 94.0            | 00:30          | x30           |
|                      | 56.0            | 00:20          |               |
|                      | 72.0            | 00:45          |               |
| Final Extension      | 72.0            | 02:00          |               |
| Hold                 | 10.0            |                |               |

**Table 2.5:** Temperature profile used for PCR of Afa-family probe

| Step                 | Temperature(°C) | Time (minutes) | No. of cycles |
|----------------------|-----------------|----------------|---------------|
| Initial denaturation | 94.0            | 03:00          |               |
| Denaturation         | 94.0            | 01:00          | x35           |
| Annealing            | 55.0            | 01:00          |               |
| Elongation           | 72.0            | 02:00          |               |
| Final Extension      | 72.0            | 05:00          |               |
| Hold                 | 10.0            |                |               |

#### **2.5.7.2 Visualisation of PCR products**

3.0 ul of 6X loading dye was added to 5 µl of the amplified product, to make the final concentration of the loading buffer in the reaction samples to 1X. The PCR products were resolved on a 1.5% superfine resolution agarose (Bioline, Molecular grade) gel. The gel was prepared in 1x TAE buffer (Appendix 1) and ethidium bromide was added at a concentration of 0.5 µg/µl. A 1Kb ladder was run along with the samples to estimate the product size. The gel was run at 80V for 40 mins and visualised under UV light and photographed using the UVP gel documentation system (Syngene, Scientific Laboratory Supplies).



### 2.5.7.3 Making probes

#### 2.5.7.3.1 Nick translation reaction (20 µl) for FISH probes:

**Table 2.6:** Nick translation reaction components and their volume in micro litre (µl) used to make FISH probes.

| Components                         | Volume (µl) |
|------------------------------------|-------------|
| ddH <sub>2</sub> O                 | 4           |
| DNA (PCR product)                  | 7           |
| 10x Nick translation buffer        | 2           |
| Non-labeled dNTPs(2mM each, mixed) | 2           |
| Labeled dNTP (1mM)                 | 0.5         |
| DNA Polymerase I (10U/µl)          | 4           |
| Dnase (100mU/µl) diluted           | 0.5         |

Each sample was gently mixed by pipetting and then incubated at 16°C for 2 hours in the dark

#### 2.5.7.3.2 Ethanol precipitation of the probe

See section 2.5.3.2 above

### 2.5.7.4 FISH

Preparation of the metaphase spreads from root tips and UV cross linking of the slides was carried out in the same way as described in sections 2.5.6 and 2.5.7 above.

FISH probe mixture was made up as follows:

|                        |                |
|------------------------|----------------|
| Hybridisation solution | - 12 µl/slide  |
| pSc119.2 (200 ng/µl)   | - 2 µl/slide   |
| Afa family (200 ng/µl) | - 1.5 µl/slide |

### Probe denaturation:

The probe mixture was denatured in a PCR machine at 85°C for 8 minutes 30 seconds and the probe then transferred directly on to ice.

The probe mix (15 µl) was added to a cover slip and the cover slip was then put on the slides which had previously been cross linked.

### Denaturation of slides:

The slides were denatured in a PCR machine at 75°C for 5 minute 30 seconds.

### Hybridisation of slides:

The slides were placed on a slide holder in a moist chamber (see section 2.5.6 above) with a closed lid. The slide box was then incubated at 38°C overnight.

### Post hybridisation washing:

The post hybridisation washing of the slides was carried out in the same way as described in section 2.3.7 above.

## **2.6 Genotyping**

### **2.6.1 Axiom® Wheat-Relative Array design**

The Axiom® Wheat/Wild relative 35K SNP array was developed in collaboration between Affymetrix and the Universities of Nottingham and Bristol. The array is composed of SNPs polymorphic between four wheat cultivars (Paragon, Chinese Spring, Highbury and Pavon 76) and the 10 wild relatives of wheat used in the introgression programme at the University of Nottingham (*Aegilops caudata*, *Aegilops speltoides*, *Amblyopyrum muticum*, *Secale cereale*, *Thinopyrum bessarabicum*, *Thinopyrum elongatum*, *Thinopyrum intermedium*, *Thinopyrum ponticum*, *Triticum timopheevii* and *Triticum urartu*).

The array was used to genotype 504 *T. timopheevii* introgression lines (see Appendix 2). DNA, diluted to a final concentration of 50 ng/μl using the formula  $N_1 \times V_1 = N_2 \times V_2$ , where N is the Normality and V is the volume and 1 and 2 are the initial and final values, was sent to the University of Bristol for genotyping on an Axiom® Wheat/Wild relative 35K SNP array. For the genotyping methods and the subsequent analysis of the genotyping see Chapter 4, section 4.2.3.

## **2.7 KASP markers**

In order to use marker assisted selection of *T. timopheevii* introgressions, KASP markers were designed. The work flow for this is outlined in Chapter 4 in material and methods (section 4.2.6).

## **2.8 Mineral Analysis**

### **2.8.1 Digestion and analysis of leaf samples**

Samples (~0.2 g DW) of leaf were digested using a microwave system comprising a Multiwave 3000 platform with a 48-vessel MF50 rotor (Anton Paar GmbH, Graz, Austria). The digestion vessels were made up of perfluoroalkoxy (PFA) liner material and polyethylethylketone (PEEK) pressure jackets (Anton Paar GmbH).

Leaf material was digested in 2 ml 70% Trace Analysis Grade (TAG) HNO<sub>3</sub>, 1 mL Milli-Q water (18.2 MΩ cm; Fisher Scientific UK Ltd, Loughborough, UK), and 1 mL H<sub>2</sub>O<sub>2</sub> with microwave settings as follows: power = 1400 W, temp = 140°C, pressure = 20 Bar, time = 45 minutes. Two operational blanks were included in each digestion run. Duplicate samples of certified reference material (CRM) of leaf (Tomato SRM 1573a, NIST, Gaithersburg, MD, USA) were included approximately every fourth digestion run. Laboratory reference material (LRM)

from pooled / freeze-dried *Brassica napus* leaves was also used for later digests. Following digestion, each tube was made up to a final volume of 15 mL by adding 11 mL Milli-Q water, transferred to a universal tube and stored at room temperature.

### **2.8.2 Inductively coupled plasma mass spectrometry (ICP-MS)**

Leaf digests were diluted 1-in-5 using Milli-Q water prior to elemental analysis. The concentrations of 28 elements were obtained using inductively coupled plasma-mass spectrometry (ICP-MS; Thermo Fisher Scientific iCAPQ, Thermo Fisher Scientific, Bremen, Germany); Silver (Ag), Aluminium (Al), Arsenic (As), Boron (B), Barium (Ba), Calcium (Ca), Cadmium (Cd), Chromium (Cr), Cobalt (Co), Cesium (Cs), Copper (Cu), Iron (Fe), Potassium (K), Magnesium (Mg), Manganese (Mn), Molybdenum (Mo), Sodium (Na), Nickel (Ni), Phosphorus (P), Lead (Pb), Rubidium (Rb), Sulphur (S), Selenium (Se), Strontium (Sr), Titanium (Ti), Uranium (U), Vanadium (V), Zinc (Zn). Operational modes included: (i) a helium collision-cell (He-cell) with kinetic energy discrimination to remove polyatomic interferences, (ii) standard mode (STD) in which the collision cell was evacuated, and (iii) a hydrogen collision-cell (H<sub>2</sub>-cell). Samples were introduced from an autosampler incorporating an ASXpress™ rapid uptake module (Cetac ASX-520, Teledyne Technologies Inc., Omaha, NE, USA) through a PEEK nebulizer (Burgener Mira Mist, Mississauga, Burgener Research Inc., Canada). Internal standards were introduced to the sample stream on a separate line via the ASXpress unit and included Sc (20 µg L<sup>-1</sup>), Rh (10 µg L<sup>-1</sup>), Ge (10 µg L<sup>-1</sup>) and Ir (5 µg L<sup>-1</sup>) in 2% trace analysis grade HNO<sub>3</sub> (Fisher Scientific UK Ltd). External multi-element calibration standards (Claritas-PPT grade CLMS-2; SPEX Certiprep Inc., Metuchen, NJ, USA) included Ag, Al, As, B, Ba, Cd, Ca, Co, Cr, Cs, Cu, Fe,

K, Mg, Mn, Mo, Na, Ni, P, Pb, Rb, S, Se, Sr, Ti (semi-quant), U, V and Zn, in the range 0 – 100  $\mu\text{g L}^{-1}$  (0, 20, 40, 100  $\mu\text{g L}^{-1}$ ). A bespoke external multi-element calibration solution (PlasmaCAL, SCP Science, Courtaboeuf, France) was used to create Ca, K, Mg and Na standards in the range 0-30  $\text{mg L}^{-1}$ . Boron, P and S calibration utilized in-house standard solutions ( $\text{KH}_2\text{PO}_4$ ,  $\text{K}_2\text{SO}_4$  and  $\text{H}_3\text{BO}_3$ ). In-sample switching was used to measure B and P in STD mode, Se in  $\text{H}_2$ -cell mode and all other elements in He-cell mode. Sample processing was undertaken using Qtegra™ software (Thermo Fisher Scientific) with external cross-calibration between pulse-counting and analogue detector modes when required.

## Chapter 3

### Germplasm production

#### 3.1 Introduction

*Triticum timopheevii* is a tetraploid wheat that has both cultivated and wild forms. The genus *Triticum* L. contains both natural and synthetic allopolyploids possessing the G genome. These include the wild species *T. araraticum*, the domesticated wheats *T. timopheevii* Zhuk. ( $2n=4x=28$ ,  $GGA^tA^t$ ) and *T. timopheevii* (Zhuk.) ssp. *armeniaceum* and a hexaploid species known as *T. zhukovskyi* ( $2n=6x=42$ ,  $GGA^tA^tA^mA^m$ ) (Dorofeev *et al.*, 1979; van Slageren, 1994).

Worldwide, more than 770 accessions of *T. timopheevii* and its close relatives are stored as seed in the national collections of Japan (Kyoto university, more than 337 accessions), USA (USDA-ARS and WGRRC, KSU-about 330) and Russia (VIR, 103) (Johnson and Waines, 1977; Kawahara, 2005).

*T. timopheevii* was first described by P. M. Zhukovsky in 1923, as *Triticum dicoccum* var. *timopheevii* Zhuk. Later, after more detailed studies, this wheat was regarded by him as a novel species under the name *T. timopheevii* (Zhuk.) (Zhukovsky, 1928).

During germplasm collection, it was found that local farmers had known this wheat for years, called it "Zandhuri" and cultivated it in their fields. From a botanical viewpoint, Zandhuri wheat was actually a mix of *T. timopheevii* and cultivated einkorn *T. monococcum*. Not only were the grains of Zandhuri wheat used for bread but also the straw was utilised as a roofing material (Dekaprelevich and Menabde, 1932).

*T. timopheevii* is a late maturing spring wheat, where the height of plants can reach 155 cm. Ears are pyramidal in shape, rather short (a length of approx. 4.7 cm), awned, dense and flat (the width considerably exceeds the thickness). The sheath and leaf blades, stem nodes, and glumes are pubescent (covered with stiff, dense, and long hairs) (Dorofeev *et al.*, 1979). It flowers from June to July and the seed ripens from August to September. Under normal conditions, the number of spikelets per ear comprises 11-15, the number of grains per ear ranges from 14 to 23, and 1000-grain weight amounts to 35.2 g (Romanov *et al.*, 2013). It is suitable for light (sandy), medium (Loamy) and heavy (clay) soils but prefers well-drained soils. The flowers are hermaphrodite (have both male and female organs) and are pollinated by wind ([www.pfaf.org](http://www.pfaf.org)).

A cultivated free threshing form with naked grain and very dense ears was discovered within the *T. timopheevii* wheat. This natural mutant form was described as an individual species in 1969 under the name *T. militinae* Zhuk. ( $2n=28$ ,  $GA^t$ ) (Zhukovsky and Migushova, 1969). The domesticated form of *T. timopheevii* was restricted to western Georgia while the wild form *T. araraticum* is found in west Iran, north Iraq, south-eastern Turkey and Transcaucasia.

### **3.1.1 Evolution, Phylogeny and Domestication**

There are two hypotheses proposed for the origin of the tetraploid wheats. The first is the monophyletic origin where it is proposed that *T. timopheevii* and *T. turgidum* originated from one hybridisation event, then diverged due to chromosomal rearrangements or introgressive hybridisation with another unknown diploid species (Gill and Chen, 1987). The second is the diphyletic hypotheses which suggests that *T. timopheevii* and *T. turgidum* originated from two separate hybridisation events. The diphyletic hypothesis is supported by the

different species-specific chromosome translocations in the two species (Jiang and Gill, 1994a).

Irregular meiosis and sterility is observed when the members of this group are crossed with other tetraploid and hexaploid species. This led to the conclusion that *T. timopheevii* has only one genome in common (Lilienfeld and Kihara, 1934). The second genome showed, at the most, only partial homology. Hence the genome constitution was proposed for *T. timopheevii* as A<sup>t</sup>G. The origin of the A genome i.e. which particular species contributed the A genome of *T. timopheevii* was not clear. All three diploid wheats, *T. monococcum* (Lilienfeld, 1951), *T. boeoticum* (Belay, 2000; Konarev *et al.*, 1976), and *T. urartu* (Dvorak, 1998; Kilian *et al.*, 2007), were proposed as possible sources of the A<sup>t</sup> genome. Studies on RFLP analysis of repetitive DNA sequences (Dvorak *et al.*, 1993), 5s rDNA studies (Allaby and Brown, 2000), and AFLP analysis of nuclear DNA in wheat and Aegilops species (Kilian *et al.*, 2007) strongly suggest that the A<sup>t</sup> genome of *T. timopheevii* has originated from *T. urartu*.

The origin of the G-genome has remained unidentified since no other diploid species in wheat or in closely related genera seem to have this genome. Significant differences between the A<sup>t</sup> and G genomes in terms of heterochromatin content, indicate that *T. timopheevii* is an allopolyploid rather than an autopolyploid species (Badaeva *et al.*, 1994a, 1986; Shang *et al.*, 1989). Later, it was suggested that the G genome is partially homologous to the B-genome and that it was derived from the B-genome through a process of structural differentiation of the chromosomes (Kostoff, 1936; Love, 1941; Sachs, 1953). Comparative cytogenetic analyses of wheat and related Aegilops species showed that *Ae. speltoides* Tausch is the most similar to the G genome in



chromosome morphology e.g. C-banding and FISH patterns using repetitive DNA sequences (Badaeva *et al.*, 1996, 1986; Giorgi and Bozzini, 1969; Jiang and Gill, 1994a; Zoshchuk *et al.*, 2007). This study was confirmed by immunochemical and electrophoretic studies of seed storage proteins and isozymes (Konarev *et al.*, 1976) and molecular markers (Dobretsov *et al.*, 2008). The close relationship of the G and S genomes, on the basis of chromosome pairing, is supported by the results of meiotic analysis in *T. timopheevii* x *Ae. speltooides* hybrids (Shands and Kimber, 1973). Mori *et al.* (1997) concluded that *Ae. speltooides* was the female parent and contributed the cytoplasm to *T. timopheevii* from studies on the mitochondrial and plastid DNA. The degree of divergence between the G and B genomes is higher than that between the A<sup>t</sup> and A genomes (Rodriquez *et al.*, 2000a; Huang *et al.*, 2002; Salina *et al.*, 2006).

### Domestication

It has been well documented that *T. timopheevii* was domesticated from the wild species *T. araraticum* (Dorofeev *et al.*, 1979; Kilian *et al.*, 2009; Mori *et al.*, 2009). Based on the current distribution of *T. araraticum* (Feldman, 2001), *T. timopheevii* could have been first cultivated in the eastern part of the Fertile Crescent, although there is some dispute as to the exact location.

Studies on the divergence of nucleotide sequences in different cereal species have been used to predict that the progenitor of *T. timopheevii* and its close relatives, evolved about 300,000-500,000 years ago (Huang *et al.*, 2002; Feldman and Levy, 2012). N.I. Vavilov hypothesized that *T. timopheevii* was first domesticated in north-eastern Turkey before being introduced into Georgia in ancient times (Vavilov, 1935). He believed that *T. timopheevii* could have been

established as a highly disease resistant species in the warm, humid climate of western Georgia. Alternatively Dorofeev *et al.* (1979) proposed that *T. timopheevii* was probably domesticated in Iraq, as they found morphological similarities between some local Iraq populations of *T. araraticum* with *T. timopheevii*. However, chloroplast DNA fingerprinting showed a closer relationship of *T. timopheevii* with populations of *T. araraticum* from southern Turkey and northern Syria (Mori *et al.*, 2009).

### **3.1.2 Phenotypic and genotypic characterization:**

Although *T. timopheevii* has been known for a long time, a lack of molecular markers meant only scarce information was available on its economically important characters. Advancement in molecular marker technology led to the identification of genes responsible for traits associated with grain quality. Some of the genes that have been identified in *T. timopheevii* are listed in Table 3.1.

**Table 3.1:** The different genes related to phenotypic traits in *T. timopheevii* and its close relatives, the chromosomes involved and the technology used to perform the studies.

| Genes                                          | Traits                                                           | Population                                                   | Chromosome                                                 | Technology                                 | Reference                                            | Remarks                                                                                                              |
|------------------------------------------------|------------------------------------------------------------------|--------------------------------------------------------------|------------------------------------------------------------|--------------------------------------------|------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------|
| <i>Br-At1</i>                                  | Brittle rachis                                                   | RIL [ <i>T. araraticum</i> x <i>T. timopheevii</i> ]         | 3A <sup>t</sup> S (7.4cM)                                  | Molecular markers                          | Rawat <i>et al.</i> , 2012                           | Locus flanked by markers <i>Xgwm2</i> and <i>Xbem44</i>                                                              |
| <i>18S-5.8S-26S rRNA</i>                       | Grain quality                                                    | -                                                            | <i>T. timopheevii</i> and <i>T. araraticum</i> chromosomes | N-banding and <i>In situ</i> hybridisation | Hutchinson and Miller, 1982<br>Jiang and Gill, 1994b | Two major loci- <i>Nor-B1</i> and <i>Nor-B2</i> mapped to 6A <sup>t</sup> S and 6GS                                  |
| <i>Bg</i>                                      | Black glume colour                                               | F2 [ <i>T. timopheevii</i> (K-38555) x <i>T. militinae</i> ] | 1A <sup>t</sup> S                                          | Molecular markers                          | Salina <i>et al.</i> , 2006                          | Gene is tightly linked with markers <i>Xgwm905</i> and <i>Xgwm1223</i>                                               |
| <i>Ne1</i> and <i>Ch2</i>                      | <i>Ne1</i> - Hybrid necrosis<br><i>Ch2</i> -Red hybrid chlorosis | <i>T. timopheevii</i>                                        | <i>Ne1</i> -5A <sup>t</sup><br><i>Ch2</i> -4G              | Aneuploid analysis                         | Tsunewaki, 1992                                      |                                                                                                                      |
| <i>Vrn-G1a</i>                                 | Vernalization                                                    | <i>T. timopheevii</i>                                        | G-genome                                                   | Molecular markers                          | Golovnina <i>et al.</i> , 2010                       | <i>VRN1</i> promoter of A <sup>t</sup> genome related to <i>VRN1</i> promoter of diploid wheats                      |
| GPC                                            | <i>Grain protein content</i> - grain quality                     | 62 ILs [ <i>T. miguschovae</i> x bread wheat]                | 2A <sup>t</sup> and 2G chromosomes                         |                                            | Zlatska <i>et al.</i> , 2008                         | GPC correlate with <i>NAM-G1</i> expression level plays role in regulating the N remobilisation from leaves to grain |
| <i>NAM-G1</i> genes and <i>NAC</i> gene family | N remobilisation and nitrogen assimilation control               | 12 <i>T. timopheevii</i> accessions                          | 6G                                                         | Cloning and Expression analysis            | Hu <i>et al.</i> , 2013                              | <i>T. timopheevii</i> possesses abundant germplasm resources for increase of grain protein content                   |

|                                     |                                    |                                                                                                          |                                                                                   |                              |                         |                                                                                                                                                                                 |
|-------------------------------------|------------------------------------|----------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------|------------------------------|-------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <i>Pina</i> and <i>Pinb</i> genes   | Wheat grain texture                | 65 accessions of <i>T. timopheevii</i> and <i>T. araraticum</i> and 3 accessions of <i>T. zhukovskyi</i> | A <sup>t</sup> -genome haplotype was maintained and the G-genome copy was deleted | Haplotype structure analysis | Li <i>et al.</i> , 2008 | Wheat grain texture is determined by hardness ( <i>Ha</i> ) locus consisting of genes <i>Puroindoline a</i> ( <i>Pina</i> ) and B ( <i>Pinb</i> ) (Giroux <i>et al.</i> , 2003) |
| <i>Wx-TtA</i> and <i>Wx-G</i> locus | Waxy protein for amylose synthesis | <i>T. timopheevii</i>                                                                                    |                                                                                   | Molecular markers            | Yan and Bhavé, 2001     | Enzyme GBSSI (granule-bound starch synthase I), also known as waxy protein is responsible for amylose synthesis (Graybosh, 1998)                                                |
| <i>5S rRNA</i> gene                 | Grain quality                      | <i>T. militinae</i>                                                                                      | Three loci mapped on 1A <sup>t</sup> S, 5A <sup>t</sup> S, 5G                     | FISH                         | Badaeva, 2001           |                                                                                                                                                                                 |

The phenotypic and genotypic characteristics listed in Table 3.1, demonstrate that *T. timopheevii* possesses considerably useful genetic variability which can be used for wheat improvement.

### **3.1.3 Gene Pool**

*T. timopheevii* (Zhuk.) and *T. araraticum* Jakubz belong to the secondary gene pool of wheat, i.e. they have genomes that are partially homologous to the wheat genomes.

### **3.1.4 Introgression of *T. timopheevii***

To introgress *T. timopheevii* genetic material into wheat, breeders use repeated backcrossing of F<sub>1</sub> interspecific hybrids with the wheat parent to overcome hybrid sterility and cytoplasmic male sterility (Brown-Guedira *et al.*, 1997; Kalinina and Budashkina, 2001). The production of substitution lines of A<sup>t</sup> and G genome chromosomes for their wheat homoeologues in a hexaploid background comes with the limiting factor that the A<sup>t</sup> and G genomes are only partially homologous with the A and B genomes. Thus, they can only pair and recombine with the wheat chromosomes in the absence of the *Ph1* gene which normally suppresses homoeologous pairing.

Analysis of introgression lines carrying genetic material of *T. timopheevii* using mainly cytogenetic or SSR markers allowed identification of chromosomal segments containing genes for various agronomically important traits such as pest resistance etc. Some of the examples where *T. timopheevii* has been successfully introgressed into wheat with many of the introgressions showing disease resistance are listed below. This work is a result of different groups working with different methodologies to introgress *T. timopheevii* into wheat.

Two major groups have focused on the wild relative *T. timopheevii* and its wild form *T. araraticum*:

- 1) The first group is a collaboration between WGRC [Wheat Genetic Resource Centre, involving Kansas State University and USDA-ARS (United State Department of Agriculture National Research Initiative)] in the United States and the University of Sydney, Australia. Gene transfers from *T. timopheevii* ssp. *timopheevii* and *T. araraticum* successfully transferred into wheat by this consortium are given in Table 3.2.

**Table 3.2:** Studies involving different chromosomes from *T. timopheevii* ssp. *timopheevii* and *T. araraticum*, showing disease resistance and their transfers into wheat.

| Species                                                     | Gene                      | Transfer to wheat | Mapped to chromosome | Method of transfer         | Germplasm             | Reference                                                                                                                      |
|-------------------------------------------------------------|---------------------------|-------------------|----------------------|----------------------------|-----------------------|--------------------------------------------------------------------------------------------------------------------------------|
| <i>T. timopheevii</i><br><i>ssp. timopheevii</i>            | <i>Sr36/Pm6</i>           | 2B                | 2G                   | Homoeologous recombination | C747                  | Allard and Shands, 1954; Friebe <i>et al.</i> , 1996; Jorgensen and Jensen, 1973; McIntosh and Luig, 1973; Nyquist, 1957, 1962 |
| <i>T. timopheevii</i><br><i>ssp. timopheevii</i>            | <i>Sr37</i>               | 4B                | 4G                   | Homoeologous recombination | Line W                | Gyrfas, 1968; McIntosh and Gyrfas, 1971; McIntosh, 1991; Dvorak, 1983                                                          |
| <i>T. araraticum</i>                                        | <i>Sr40</i>               | 2B                | 2G                   | Homoeologous recombination | RL6087                | Dyck, 1992                                                                                                                     |
| <i>T. timopheevii</i><br><i>ssp. timopheevii</i>            | <i>Lr18</i>               | 5B                | 5G                   | Homoeologous recombination | Thatcher/ <i>Lr18</i> | Yamamori, 1994; McIntosh, 1983                                                                                                 |
| <i>T. timopheevii</i><br><i>ssp. armeniacum</i><br>(TA 870) | <i>Lr50</i>               | 2B                | 2G                   | Direct crossing            | KS96WGRC36            | Brown-Guedira <i>et al.</i> , 1999a, 2003                                                                                      |
| <i>T. timopheevii</i><br><i>ssp. armeniacum</i><br>(TA 28)  | Leaf rust resistance      | -                 | -                    | Direct crossing            | KS96WGRC35            | Brown-Guedira <i>et al.</i> , 1999a, 2003                                                                                      |
| <i>T. timopheevii</i><br><i>ssp. armeniacum</i><br>(TA 895) | Powdery mildew resistance | -                 | -                    | Direct crossing            | KS96WGRC37            | Brown-Guedira <i>et al.</i> , 1999b                                                                                            |
| <i>T. timopheevii</i><br><i>ssp. armeniacum</i><br>(TA 895) | Tan spot resistance       | -                 | -                    | Direct crossing            | KS96WGRC38            | Brown-Guedira <i>et al.</i> , 1999c                                                                                            |

|                                                                |               |     |   |                 |            |                                    |
|----------------------------------------------------------------|---------------|-----|---|-----------------|------------|------------------------------------|
| <i>T. timopheevii</i><br><i>ssp. armeniacum</i><br>(TA 960)    | FHB           | -   | - | Direct crossing | KS96WGRC46 | Brown-Guedira <i>et al.</i> , 2005 |
| <i>T. timopheevii</i><br><i>ssp. armeniacum</i><br>(PI 427315) | <i>Pm37</i>   | 7AL | - | Amphiploid      | NCAG11     | Perugini <i>et al.</i> , 2008      |
| <i>T. timopheevii</i><br><i>ssp. armeniacum</i><br>(PI 538457) | <i>MIAG12</i> | 7AL | - | Amphiploid      | NCAG12     | Maxwell <i>et al.</i> , 2009       |



The mode of transfer followed by this group has mainly been to use homoeologous recombination between wheat chromosomes and alien chromosomes, chromosome arms, or chromosome segments. The stem rust resistance gene *Sr36* was transferred to wheat chromosome 2B and was found to be closely linked to powdery mildew resistance gene *Pm6*. With the help of SSR markers *Sr36* was mapped on the short arm and *Pm6* gene was mapped on the long arm of chromosome 2G (Qin *et al.*, 2011; Wu *et al.*, 2009). The *Sr36/Pm6* gene complex was also found to be resistant to black point, crown rot and showed increased grain protein (Lehmensiek *et al.*, 2008). *Sr37* was transferred to wheat chromosome 4B and was located on chromosome 4G present in a 4G(4B) substitution line (SrTt-2) (Dvorak, 1983). The leaf rust resistance gene *Lr18* was transferred to wheat chromosome 5B (McIntosh, 1983), Yamori (1994) found a terminal N-band marker which was thought to be associated with the *Lr18* gene on 5GL. The stem rust resistance gene *Sr40*, was transferred to chromosome arm 2BS (Dyck, 1992) from two accessions of *T. araraticum* accession PGR6126 and PGR6195.

A different approach of crossing previously generated amphihaploids or a backcross line directly with wheat cultivar followed by selfing was also followed by some studies (Perugini *et al.*, 2008; Maxwell *et al.*, 2009).

2) The second group which has focused on *T. timopheevii* is a joint collaboration between the Institute of Cytology and Genetics, the Vavilov Institute of General Genetics, Ehgelhardt, and the Institute of Molecular Biology, which are all part of the Russian Academy of Sciences, Russia, the Leibniz Institute of Plant Genetics and Crop Plant Research, Gatersleben, Germany and the Agricultural Research Institute of the Hungarian Academy, Hungary. Introgression lines found to be a

source of pathogen resistance gene such as leaf rust and other agronomically useful traits are given in Table 3.3

**Table 3.3:** Studies on different introgression lines involving range of chromosomes from *T. timopheevii* ssp. *viticulosum* in particular showing disease resistance and other useful traits.

| Species                                       | Chromosomes                    | Characteristics                                                                                                                   | Reference                           | Methods of production                                                                                                |
|-----------------------------------------------|--------------------------------|-----------------------------------------------------------------------------------------------------------------------------------|-------------------------------------|----------------------------------------------------------------------------------------------------------------------|
| <i>T. timopheevii</i> ssp. <i>viticulosum</i> | Loci on chromosomes 5B and 2A  | Leaf rust resistance                                                                                                              | Leonova <i>et al.</i> , 2011        | Introgression lines obtained by backcrossing once followed by selfing                                                |
| <i>T. timopheevii</i> ssp. <i>viticulosum</i> | Introgression in 2A, 2B and 5A | Increase in transpiration and stomatal conductance, decrease in water use efficiency and drought tolerance                        | Pshenichnikova <i>et al.</i> , 2016 | Introgression line 821 obtained by backcrossing                                                                      |
| <i>T. timopheevii</i> ssp. <i>viticulosum</i> | Introgression in 2A and 5B     | 2A increased while 5B decreased the wheat tolerance to salinization                                                               | Yudina <i>et al.</i> , 2016         | Introgression lines obtained by backcrossing                                                                         |
| <i>T. timopheevii</i> ssp. <i>viticulosum</i> | Introgression of 2GL           | Positive influence on number of grains in main ear, number of spikelet per ear, decrease in plant length and stem rust resistance | Timonova <i>et al.</i> , 2012b      | Introgression lines obtained by backcrossing once followed by selfing                                                |
|                                               | Introgression of 5G            | Resistance to leaf rust in western Siberia and stem rust for Omsk oblast                                                          |                                     |                                                                                                                      |
| <i>T. timopheevii</i>                         | 6B-6G translocation chromosome | <i>Pm27</i> gene                                                                                                                  | Jarve <i>et al.</i> , 2000          | Introgression line 146-155-T produced by backcrossing                                                                |
|                                               | 6B(6G) substitution lines      | Resistance to leaf rust, brown rust and powdery mildew                                                                            | Uhrin <i>et al.</i> , 2012          | Substitution line produced by crossing amphiploid Fleischmann 481 with <i>T. timopheevii</i> ssp. <i>timopheevii</i> |

|                                                  |                                                                       |              |                              |                                                                                                                                               |
|--------------------------------------------------|-----------------------------------------------------------------------|--------------|------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------|
| <i>T. timopheevii</i> ssp.<br><i>viticulosum</i> | Chromosome 2A tightly linked with<br><i>Xgwm817</i>                   | <i>LrTt1</i> | Leonova <i>et al.</i> , 2004 | Line 842 (single<br>introgression fragment on<br>chromosome 2A and 2B)<br>used for mapping produced<br>by backcrossing followed by<br>selfing |
|                                                  | Chromosome 5G linked to markers<br><i>Xgwm814</i> and <i>Xgwm1257</i> | <i>LrTt2</i> | Leonova <i>et al.</i> , 2010 | Line 842-2 (sister line of<br>Line 842)                                                                                                       |

The mode of producing introgression lines followed by this group was largely based on direct crosses between wheat and *T. timopheevii* where only one species *T. timopheevii* ssp. *viticulosum* was mostly targeted. Following the production of introgression lines, sterile F<sub>1</sub> hybrids were backcrossed to the initial wheat cultivar with subsequent self-pollination. Selection in backcross generations was then performed for cytologically stable resistant plants, which served as the basis for the development of introgression lines. Another approach of crossing amphihaploids directly with wheat cultivars followed by backcrossing and selfing was also followed in some studies (Leonova *et al.*, 2004, 2010; Jarve *et al.*, 2000; Uhrin *et al.*, 2012).

The development of large numbers of introgression lines and hybrids between wheat and *T. timopheevii* and *T. araraticum* and the range of traits introgressed suggests that further introgression work with *T. timopheevii* has enormous potential for wheat improvement. The studies discussed above do not report on the crossability of *T. timopheevii* with wheat or the fertility rates.

Both consortiums have therefore use the same methods to transfer genetic variation from *T. timopheevii* and derivatives into wheat.

In the past specific accessions or addition or substitution lines were targeted for introgression of *T. timopheevii* into wheat (discussed above in Table 3.2 and 3.3) because they carry genetic variation for a specific agronomically important target trait e.g. disease resistance. The drawback with this strategy was that while it led to the introgression of genetic variation for the target trait, other introgressions carrying important genetic variation for a vast range of other agronomically important traits were generally lost. Hence, the main aim of this work using several accessions available at the Wheat Research Centre (WRC,

Nottingham) and not targeting particular accessions and addition or substitution lines is to transfer as many chromosome segments of *T. timopheevii* to wheat (var. Paragon) using a whole genome transfer approach (King *et al.*, 2013) and then to screen for as wide a range of agronomically important traits as possible i.e. to exploit any potentially important genetic variation from *T. timopheevii* to the full.

### **3.1.5 Why *T. timopheevii* has not been exploited**

Although *T. timopheevii* possesses useful genetic variability and is resistant to many fungal diseases and pests, its potential has not been sufficiently exploited for the improvement of hexaploid wheat. Gene transfer from distantly related species to wheat is a challenging task which depends to a great extent on the degree of divergence between the genomes of the species involved in the crosses. The other major factor which may complicate the introgression of *T. timopheevii* genetic material to wheat is a lack of molecular technologies to allow the detection and Characterization of introgressed segments. Earlier work depended very much on phenotyping.

### **3.1.6 Aims and objectives of this chapter**

The main aims of this chapter are-

- 1) Present the development/generation of introgression lines between wheat (var. Paragon) and all the *T. timopheevii* accessions listed in Table 3.4
- 2) Analyse the level of fertility between different accessions of *T. timopheevii* in crosses to wheat and in different generations through the backcross generations

## **3.2 Material and methods**

### **3.2.1 Plant material**

The hexaploid wheat varieties used were Paragon and Chinese Spring (both euploid and *ph1* mutant) while six accessions of *T. timopheevii* were used, all obtained from the USDA genebank (Table 3.4). Photos of four of these accessions are shown in Figure 3.1. Details of the collection sites of all the accessions of *T. timopheevii* used are given in Table 3.4.

**Figure 3.1:** Four of the six wild accessions (538512, 289752, 355452 and P95-99.1-1) of *T. timopheevii* that were used for crossing.



538512



289752



355452



P95-99.1-1



**Table 3.4:** Accessions of *T. timopheevii* (and ssp.) used in the introgression programme and original collection sites (where the data is available).

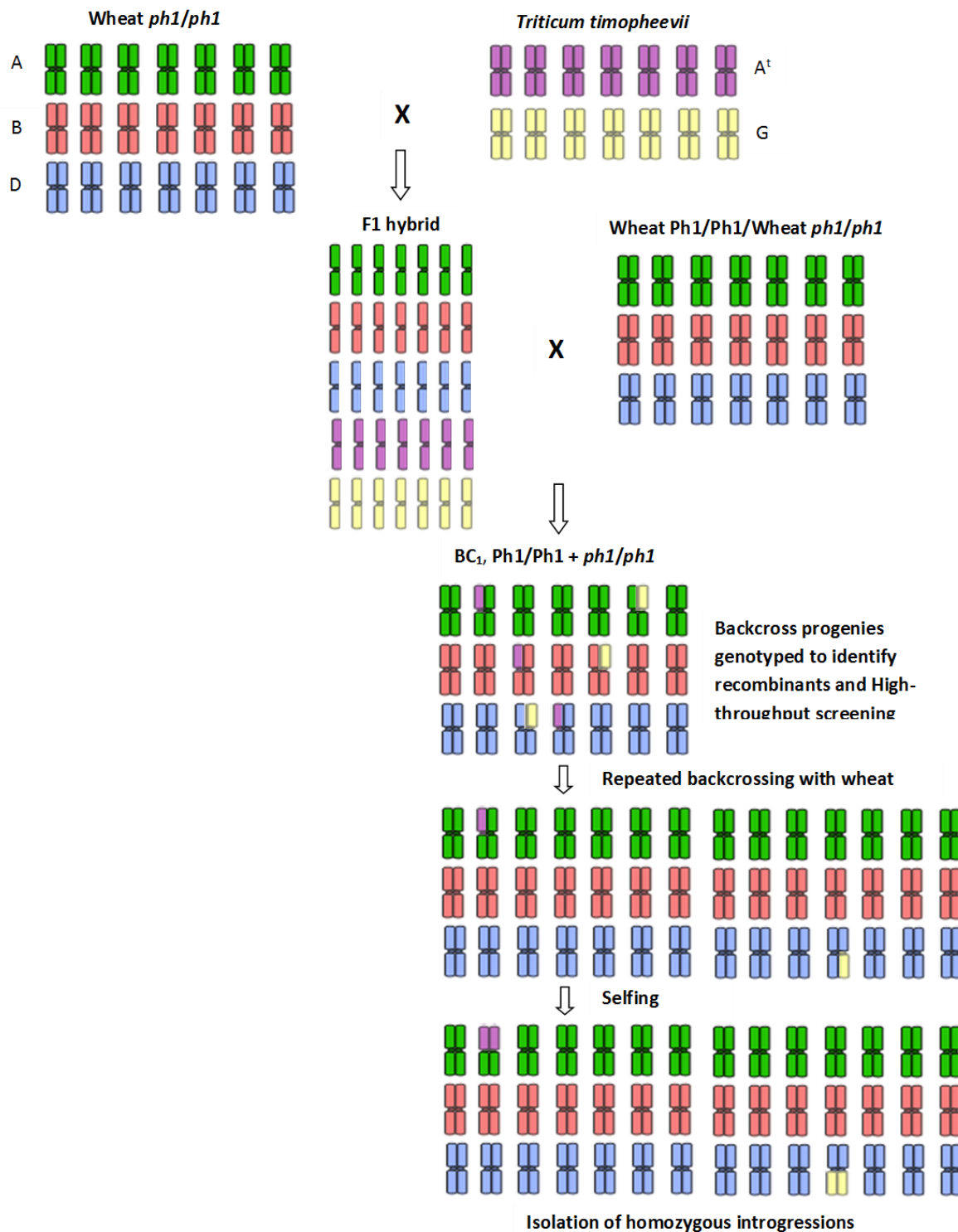
| Species               | Subspecies         | Accessions | Collection site            |
|-----------------------|--------------------|------------|----------------------------|
| <i>T. timopheevii</i> | <i>timopheevii</i> | P95-99.1-1 | USDA, site unknown         |
| <i>T. timopheevii</i> | <i>timopheevii</i> | 289752     | Australia, New South Wales |
| <i>T. timopheevii</i> | <i>armeniicum</i>  | 427414     | Iraq, Dahuk                |
| <i>T. timopheevii</i> | <i>armeniicum</i>  | 427998     | Lebanon, El Beqaa          |
| <i>T. timopheevii</i> | <i>armeniicum</i>  | 538512     | Iraq, As Sulaymaniyah      |
| <i>T. timopheevii</i> | NA                 | 355452     | Azerbaijan, Naxcivan       |

Accession P95-99.1-1 was used for the majority of the backcrossing programme

### 3.2.2 Crossing Programme

The crossing programme for the production of *T. timopheevii* introgressions into a hexapod wheat background (var. Paragon) is shown in Figure 3.2.

**Figure 3.2:** Crossing scheme for the production of *T. timopheevii* introgressions into wheat (var. Paragon) where *T. timopheevii* is used as male parent to their respective wheat parent (var. Paragon, *Ph1* mutant) for the production of F<sub>1</sub> interspecific hybrid. The F<sub>1</sub>, BC<sub>1</sub> hybrid used as female parent was crossed to wheat (var. Paragon, euploid and *Ph1* mutant) to produce successive generations. Once the recombinants were identified, they were selfed to produce the homozygous introgressions.



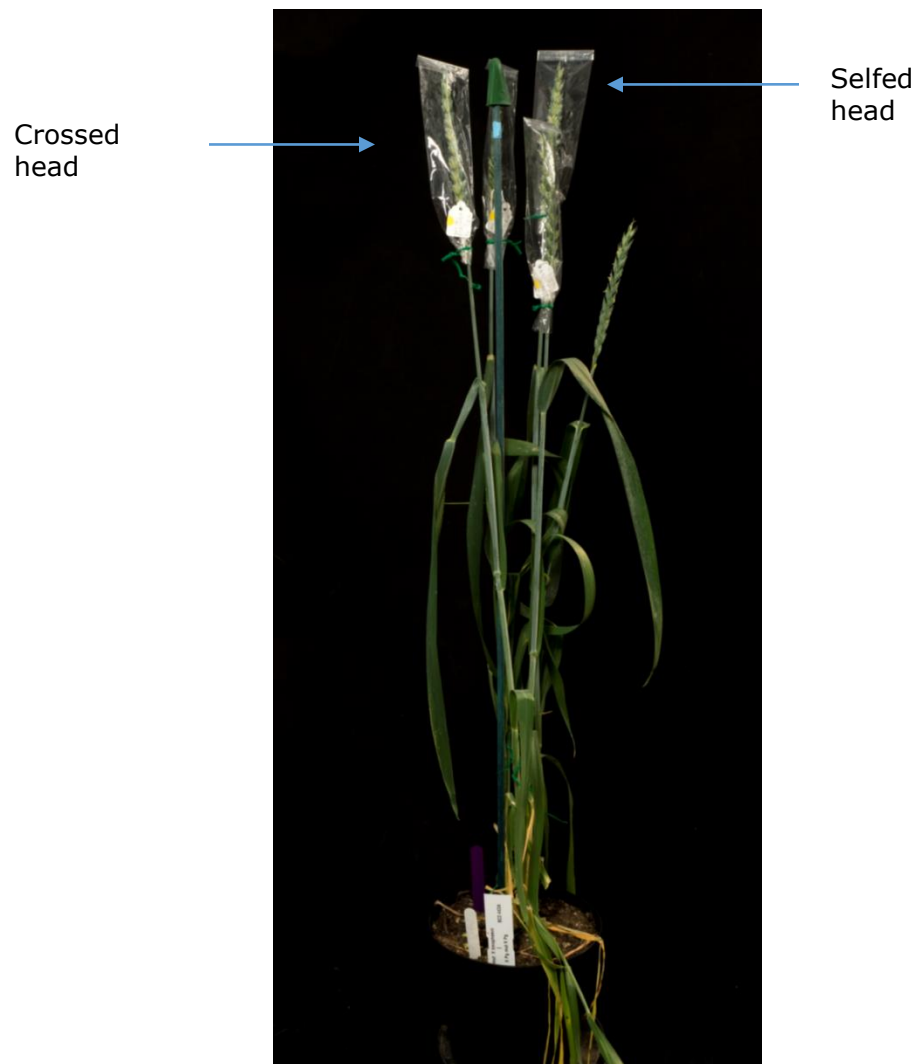
#### a) **Production of F<sub>1</sub> interspecific hybrids**

*T. timopheevii* was used as the male parent to pollinate emasculated spikes of Paragon *ph1* mutant to produce F<sub>1</sub> hybrid seed. Figure 3.3 shows the head morphology of *T. timopheevii*, Chinese Spring *ph1* mutant, F<sub>1</sub> hybrid and further backcross generations. The tertiary, etc. florets of each spikelet were removed i.e. only the primary and secondary spikelets of each floret were retained for emasculature. Emasculature was carried out 1-3 days prior to anthesis (before natural self-pollination occurred). Each emasculated head was covered with a glassine bag to prevent uncontrolled pollination. Florets were pollinated with *T. timopheevii* pollen once the stigmas were judged to have become receptive i.e. the stigma got fluffy, and after pollination all heads were again covered with glassine bags. Figure 3.4 shows a BC<sub>2</sub> plant with crossed heads.

**Figure 3.3:** Heads of Chinese Spring *ph1* mutant and *T. timopheevii* (P95-99.1-1) crossed resulting in F<sub>1</sub>, BC<sub>1</sub> and BC<sub>2</sub> heads of *T. aestivum*/*T. timopheevii* where F represents female parent and M male parent.



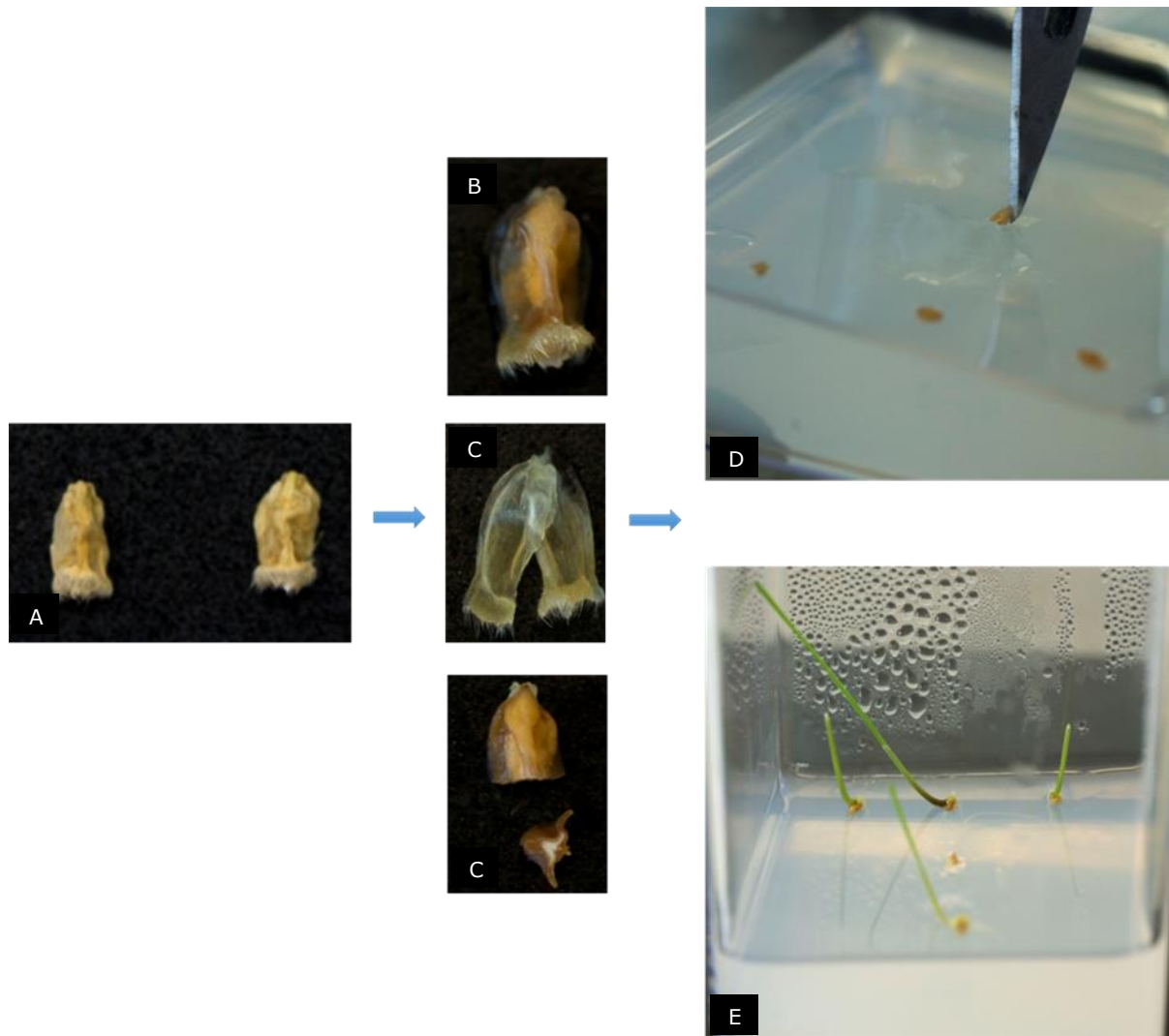
**Figure 3.4:** Backcross plant (BC<sub>2</sub>-443A) of *T. timopheevii* showing crossed heads and self-head covered with glassine bags.



## **b) Embryo culture**

All the F<sub>1</sub> seed produced were small and shrivelled and hence germination rate was poor. Therefore to increase the number of seeds germinating, embryo culture was used. F<sub>1</sub> seeds from crosses were sterilised with 1ml 5% CNaO (Appendix 1). The seeds were then transferred to sterile water for 2 days. Germinated seeds were sown directly into soil in trays. Seeds which did not germinate were embryo rescued and the embryos placed on culture medium (MS + 2% sucrose + 1% agarose, Appendix 1). Figure 3.5 shows the shrivelled grain and steps in the culture of some of these F<sub>1</sub> seeds. Germinated F<sub>1</sub> embryos were transferred to soil in trays. New F<sub>1</sub> seedlings were kept in a vernalisation chamber (6°C) for 8 weeks. Seedlings were then transferred into pots and plants were grown in a glasshouse (16 hours light at 20°C and 8 hours dark at 15°C).

**Figure 3.5:** Shrivelled grain culture a) shrivelled grains, b) seeds treated with 1ml 5% CInaO, c) embryo dissection, d) embryo placed on culture medium (MS + 2% sucrose + 1% agarose), e) germinated seedlings.



### **c) Production of backcrossing 1 generation**

All F<sub>1</sub> hybrid plants were male-sterile and were backcrossed as the female parent to wheat var. Paragon (Figure 3.3) to generate the BC<sub>1</sub> seed. Central florets of each spikelet were removed before pollination and spikelets were cut just above the top of the stigma; anthers, none of which produced viable pollen, were not removed. Each emasculated head was covered with a glassine bag to prevent uncontrolled pollination. After 1 or 2 days, florets were pollinated with wheat (var. Paragon) pollen once the stigmas became receptive i.e. fluffy, and after pollination all heads were again covered with glassine bags.

### **d) Production of later backcross generations**

BC<sub>1</sub>, BC<sub>2</sub>, BC<sub>3</sub>, BC<sub>4</sub> seeds were germinated and planted in the same way as described above. Hybrid plants were grown to maturity in the glasshouse. All backcross plants (BC<sub>1</sub>, BC<sub>2</sub>, BC<sub>3</sub>, BC<sub>4</sub> etc.), whether male fertile or sterile were used as the female parent in backcrosses to the respective wheat parent (var. Paragon). The first emerged spike of each BC<sub>1</sub> plant was covered with a glassine bag prior to anthesis to prevent outcrossing, and allowed to self-pollinate (Figure 3.4 shows a BC<sub>2</sub> plant with selfed head and crossed heads) to produce self-seeds. Primary and secondary florets of each spikelet were emasculated and florets were cut just above the top of the stigma. Emasculated spikes were covered with a glassine bag until judged ready for pollination. Pollination was carried out using wheat (var. Paragon).



### Selection of seeds

Seed was randomly selected at each generation for germination as genotyping was not available at this stage. However, at each generation it was the aim to select seed tracing back to as many of the BC<sub>1</sub> plants as possible.

### **e) Observations**

Various observations of backcross seed set for each cross were made and recorded, which is stated below:

$$1. \quad \text{Per cent germination} = \frac{\text{Total number of seed germinated}}{\text{Total number of seed sown}} \times 100$$

$$2. \quad \text{Per cent seed set} = \frac{\text{Number of plants setting seed}}{\text{Number of seed germinated}} \times 100$$

$$3. \quad \text{Average number of seed set} = \frac{\text{Total number of seed produced}}{\text{Total number of crosses}}$$

$$4. \quad \text{Fertility (\% crosses set seed)} = \frac{\text{Number of crosses producing seed}}{\text{Total number of crosses done}} \times 100$$

### 3.3 Results

A total of 424 crosses were made to produce 150 F<sub>1</sub> hybrids between *T. timopheevii* (P95-99.1-1) and Paragon *ph1* mutant. Further, a total of 351, 204, 641, 312 and 15 crosses were made to produce 152 BC<sub>1</sub>, 1317 BC<sub>2</sub>, 6398 BC<sub>3</sub>, 4637 BC<sub>4</sub> and 229 BC<sub>5</sub> hybrid seeds. A total of three crossings seasons were aimed for the first two years (2014-2015) where the first season was in January, second in April and third in September. For accession 289752, a total of 16 and 26 crosses were made to produce 78 F<sub>1</sub> and 53 BC<sub>1</sub> hybrid seeds. For accession 355452, a total of 14 and 47 crosses were made to produce 18 F<sub>1</sub> and 82 BC<sub>1</sub> hybrid seeds. Further, a total of 5 and 36 crosses were made to produce 13 F<sub>1</sub> and 12 BC<sub>1</sub> seeds for accession 538512. For both accessions 427414 and 427998 a total of 8 and 1 crosses were made to produce 21 and 8 F<sub>1</sub> hybrid seeds between *T. timopheevii* and Paragon *ph1* mutant.

The number of seed produced by crossing the different accessions of *T. timopheevii* used in this study with wheat *ph1* mutant is given in Table 3.5 and 3.6. For accession P95-99.1-1 the average number of seed set per crossed ear was 0.3% while for accessions 289752, 355452, 538512, 427414 and 427998 it was 4.9%, 1.3%, 2.6%, 2.6% and 8% respectively.

A total of 12,883 seeds were produced with respect to a total of 1,947 crosses made with accession P95-99.1-1. The number of seed sown, germination rate, number of crosses, number of crosses producing seed, etc. for the backcrossing programme using *T. timopheevii* accession P95-99.1-1 are summarised in Table 3.5. The F<sub>1</sub> interspecific hybrids showed the highest germination rate (84.9%) as

compared to BC<sub>1</sub>, BC<sub>2</sub>, BC<sub>3</sub> and BC<sub>4</sub> generations- 42.2%, 77.5%, 81.2%, 66.7% respectively. In order to estimate the fertility of each backcross generation, the number of crossed ears that produced seed was recorded. Only 16.2 % of the F<sub>1</sub> crossed ears produced seed as compared with 93.6%, 92.5%, 99% and 100% from the crossed ears of BC<sub>1</sub>, BC<sub>2</sub>, BC<sub>3</sub> and BC<sub>4</sub> generations respectively.

Of the 73 F<sub>1</sub> seed germinated, only 25 reached maturity and set seed (Table 3.5). A total of 152 BC<sub>1</sub> seeds were generated from these 25 F<sub>1</sub> plants. Ninety of the 152 seeds were randomly selected and germinated, 35 survived to maturity and set seed. In total, 1317 BC<sub>2</sub> seeds were obtained from 204 crosses onto these 35 BC<sub>1</sub> plants. One hundred and eighty seven of these BC<sub>2</sub> seeds were then selected at random and germinated and subsequent pollinations yielded 6398 BC<sub>3</sub> seed. One hundred and one of these BC<sub>3</sub> seed were again selected at random and crossed to generate 4637 BC<sub>4</sub> seeds. Only six seeds from these BC<sub>4</sub> plants were further selected at random and crossed to produce 229 BC<sub>5</sub> seeds from 15 crosses onto these BC<sub>4</sub> plants (Table 3.5).

The number of heads that produced self-fertilised seed also increased in succeeding generations (Table 3.5). Thus 64.1%, 77.7%, 79.8% and 71.4% of the heads allowed to self on BC<sub>1</sub>, BC<sub>2</sub>, BC<sub>3</sub> and BC<sub>4</sub> plants respectively produced seed leading to the production of 395 BC<sub>1</sub>F<sub>1</sub>, 3077 BC<sub>2</sub>F<sub>2</sub>, 3471 BC<sub>3</sub>F<sub>1</sub> and 75 BC<sub>4</sub>F<sub>1</sub> seeds. No self-seed was produced from the F<sub>1</sub> plants as these were all male sterile.

Five further accessions of *T. timopheevii* (listed in Table 3.6), were also used to produce F<sub>1</sub> hybrid seed. With three of these accessions, some of the F<sub>1</sub> seed produced was germinated and crossed to wheat (var. Paragon, *Ph1* mutant) to

produce BC<sub>1</sub> seed. Table 3.6 summarises the number of seeds, crosses, etc for these five accessions.

The highest number of F<sub>1</sub> seed (78 from 16 crosses) was obtained with accession 289752. For accessions 427414, 355452, 538512 and 427998 the number of F<sub>1</sub> seed obtained was 21(8 crosses), 18 (14 crosses), 13 (5 crosses) and 8 (1 cross) respectively.

Out of these five accessions, the fertility was found to be highest for accession 289752 as 80.8% of the crosses produced BC<sub>1</sub> seed followed by 68.1% and 22.2% for accessions 355452 and 538512, all of which were higher than the 16.2% achieved with accession P95-99.1-1. A total of 82 BC<sub>1</sub> seeds from accession 355452, 53 BC<sub>1</sub> seeds from 289752 and 12 BC<sub>1</sub> seeds from 538512 were obtained.

Head morphology was more like cultivated wheat (cv. Paragon) as the backcross number progressed (Figure 3.3). Due to restoration of more wheat in the background of the backcrosses the crosses were more towards wheat which ultimately lift the breeding value of the *T. timopheevii*/*T.aestivum* material.

**Table 3.5:** Number of seed produced and germinated in relation to number of crosses carried out for each generation of the introgression program for *T. timopheevii* accession P95-99.1-1 into wheat.

|                                                                                                 | Wheat x <i>T. timopheevii</i> | F <sub>1</sub>   | BC <sub>1</sub>   | BC <sub>2</sub>    | BC <sub>3</sub>  | BC <sub>4</sub>  | Totals         |
|-------------------------------------------------------------------------------------------------|-------------------------------|------------------|-------------------|--------------------|------------------|------------------|----------------|
| No. of seed sown                                                                                | N/A                           | 86               | 90                | 187                | 101              | 6                | 470            |
| No. of seed germinated (%)                                                                      | N/A                           | 73<br>(84.9)     | 38<br>(42.2)      | 145<br>(77.5)      | 82<br>(81.2)     | 4<br>(66.7)      | 342            |
| No. of plants setting seed (%)                                                                  | N/A                           | 25<br>(34.2)     | 35<br>(92.1)      | 137<br>(94.5)      | 77<br>(93.9)     | 4<br>(100)       | 278            |
| No. of seed/ total number of crosses (Average number of seed set per crossed ear)               | 150/424<br>(0.3)              | 152/351<br>(0.4) | 1317/204<br>(6.5) | 6398/641<br>(10.0) | 4637/312<br>(15) | 229/15<br>(15.3) | 12883/19<br>47 |
| No. of crosses producing seed (%)                                                               | 20<br>(4.71)                  | 57<br>(16.2)     | 191<br>(93.6)     | 593<br>(92.5)      | 307<br>(99.0)    | 15<br>(100)      | 1183           |
| Number of self fertilised seed produced/ Total number of ears (Average number of seed per head) |                               | 0                | 395/39<br>(10.1)  | 3077/220<br>(14)   | 3471/124<br>(28) | 75/7<br>(10.7)   | 7018/390       |
| Number of ears producing seed/Total number of selfed heads                                      |                               | 0                | 25<br>(64.1)      | 171<br>(77.7)      | 99<br>(79.8)     | 5<br>(71.4)      | 300            |

**Table 3.6:** Number of seed produced in relation to number of crosses carried out for each generation of the introgression programme for *T. timopheevii* accessions 289752, 355452, 538512, 427414 and 427998 into wheat.

| <i>T.<br/>timopheevii</i>                                                         | Accession<br>289752                   |                | Accession<br>355452                   |                | Accession<br>538512                   |                | Accession<br>427414                   | Accession<br>427998                   |
|-----------------------------------------------------------------------------------|---------------------------------------|----------------|---------------------------------------|----------------|---------------------------------------|----------------|---------------------------------------|---------------------------------------|
|                                                                                   | Pg mut.x<br><i>T.<br/>timopheevii</i> | F <sub>1</sub> | Pg mut.x<br><i>T.<br/>timopheevii</i> | F <sub>1</sub> | Pg mut.x<br><i>T.<br/>timopheevii</i> | F <sub>1</sub> | Pg mut.x<br><i>T.<br/>timopheevii</i> | Pg mut.x<br><i>T.<br/>timopheevii</i> |
| Number of seed sown                                                               | NA                                    | 5              | NA                                    | 11             | NA                                    | 6              | NA                                    | NA                                    |
| Number of seed germinated (%)                                                     | NA                                    | 4<br>(80)      | NA                                    | 11<br>(100)    | NA                                    | 6<br>(100)     | NA                                    | NA                                    |
| Number of plants setting seed (%)                                                 | NA                                    | 4<br>(100)     | NA                                    | 8<br>(72.7)    | NA                                    | 4<br>(66.6)    | NA                                    | NA                                    |
| Number of seed/ Total number of crosses (Average number of seed per crossed head) | 78/16<br>(4.9)                        | 53/26<br>(2)   | 18/14<br>(1.3)                        | 82/47<br>(1.7) | 13/5<br>(2.6)                         | 12/36<br>(0.3) | 21/8<br>(2.6)                         | 8/1<br>(8)                            |
| Number of crosses producing seed (%)                                              | 10<br>(62.5)                          | 21<br>(80.8)   | 3<br>(21.4)                           | 32<br>(68.1)   | 5<br>(100)                            | 8<br>(22.2)    | 6<br>(75)                             | 1<br>(100)                            |

### 3.4 Discussion

Whole-genome transfer of *T. timopheevii* into wheat (var. Paragon) is being undertaken in this programme. The rationale behind this is, that although targeted transfer for a specific trait is very valuable, it is recognized that the alien chromatin might also carry genetic variation for a range of other target traits, many of which might not be predictable. Hence the production of a series of overlapping wild relative introgressions will allow for the testing in field trials and trait analysis in multiple environments. Genes conferring resistance to eyespot disease (*PchI*) and powdery mildew (*Pm*) from *Ae. ventricosa* were successfully transferred to wheat (Delibes *et al.*, 1977, 1981, 1987; Doussinault *et al.*, 1983, Garcia-Olmedo *et al.*, 1984). The lines developed were further exploited as they were found to contain other useful agronomic traits e.g. cereal cyst nematode resistances genes *Cre2* (Delibes *et al.*, 1993) and *Cre6* (Ogbonnaya *et al.*, 2001) and Hessian fly resistance gene *H27* (Delibes *et al.*, 1997).

The F<sub>1</sub> hybrids were developed using Paragon *ph1*. Paragon is a hexaploid UK spring wheat cultivar. This line was chosen as it had been a very successful elite line in the UK environment (Wingen *et al.*, 2017), suited to trait analysis (Moore, 2014), widely crossable to a range of wild relative species (Moore, 2014) and was already a key parent for the Wheat Genetic Improvement programme (WGIN-<http://www.wgin.org.uk>) with resources including EMS and  $\gamma$  mutant populations developed in WGIN programme. Ye *et al.* (1997) stated that when wheat is used as the female parent, the success of the cross is highly dependent on the wheat

cultivar. Hence it was essential in this programme to use a wheat cultivar known to be crossable to a range of wheat wild relatives species.

In order to induce wheat-wild relative homoeologous recombination, *T. timopheevii* was first crossed to *ph1* mutant lines, i.e., lines that lack the *Ph1* locus (Figure 3.2). Homoeologous recombination was expected to occur at meiosis in the female gametes of the F<sub>1</sub> interspecific hybrid. It was hypothesized that because of the absence of homologous chromosomes (and hence the only recombination possible was between homoeologous chromosomes of the A, B and D genomes of wheat and A<sup>t</sup> and G genomes of *T. timopheevii*), this strategy might lead to an enhanced frequency of introgression. The A<sup>t</sup> genome of *T. timopheevii* is thought to originate from *T. urartu* (Dvorak, 1998; Allaby and Brown, 2003; Kilian *et al.*, 2007), hence being quite similar to the A genome of wheat which is also thought to originate from *T. urartu* (Feldman and levy, 2005). The G genome of *T. timopheevii*, however while related to the B genome of wheat is not thought to be so similar (Kostoff, 1936; Love, 1941; Sachs, 1953). Thus, it was predicted that a higher level of recombination would occur between the wheat A genome chromosomes and those of the A<sup>t</sup> genome of *T. timopheevii*, than between the wheat B chromosomes and the G genome of *T. timopheevii*. Marcussen *et al.* (2014) suggested that the B genome of *Ae. sharonensis* shares a considerable similarity to the D genome of wheat. Thus as the G genome of *T. timopheevii* is thought to have evolved from a B genome donor, it is therefore possible that some recombination might also occur between the D genome chromosomes of wheat and the G genome chromosomes of *T. timopheevii*.



It has been proposed that a gene located on chromosome 5G of *T. timopheevii* homoeologous to the pairing suppressor *Ph1* on the long arm of chromosome 5B in *T. aestivum*, controls the diploid-like chromosome pairing (Feldman, 1966; Martinez *et al.*, 1996). This means that chromosome 1A<sup>t</sup> must pair with 1A<sup>t</sup> at meiosis and not with 1G. Therefore even with the use of a wheat line without the *ph1* gene in the crossing programme, the F<sub>1</sub> hybrid will carry the *T. timopheevii* copy of the *ph1* gene. The BC<sub>1</sub> populations were shown to contain wheat-alien introgressions (see chapter 4), which might be an indicator that the effect of the *ph1* gene from *T. timopheevii* is less than that of the wheat *ph1* gene and therefore does not prevent homoeologous recombination to the same extent. This might also be explained by the *T. timopheevii* *ph1* gene being less effective in a hybrid background. This was supported by Shnaider (Enno). (1989). While studying the crosses between a CS *ph1* mutant line and *T. timopheevii*, he found that the average number of bivalents were higher than in crosses between CS and *T. timopheevii*, showing that the homoeologous pairing suppressor gene on chromosome 5G of *T. timopheevii* is not fully capable of replacing *ph1* of wheat. Ozkan and Feldman. (2001) also supported the fact that the *ph1* allele of *T. timopheevii* is less effective in preventing homoeologous chromosome pairing.

Accession P95-99.1-1 (*T. timopheevii* ssp. *timopheevii*) has been the most extensively used in this project. This was primarily because it was the first accession obtained from the USDA seed bank. Miko *et al.* (2013) analyzed 56 accessions of *T. timopheevii* for agronomically valuable traits and virus infections and found that individuals of *T. timopheevii* ssp. *timopheevii* tended to be more

advantageous for wheat breeding than *T. timopheevii* ssp. *armeniicum*. Although ssp. *armeniicum* had relatively early heading and flowering dates, they were more susceptible to fungal and viral diseases, and had a prostrate growth form with lower yielding ability, which thus makes their utilization in wheat breeding more difficult. Thus P95-99.1-1 (*T. timopheevii* ssp. *timopheevii*) could be advantageous in our crossing programme and was later found to be highly resistant to Fusarium head blight (FHB) (see Chapter 6).

Due to a different ploidy level (Paragon is hexaploid and *T. timopheevii* is tetraploid) and the genomic structure (i.e. A<sup>t</sup> and G genomes are only partially homologous with the A and B genomes of wheat), the production of hybrid seeds in crosses of *T. aestivum* with *T. timopheevii* is extremely low (Shands, 1941; Allard and Shands, 1954; Skurygina, 1958; Leontjev, 1980; Kobylanskyi and Fadeeva, 1986). In crosses between wheat (var. Paragon, *ph1* mutant) as the female parent and *T. timopheevii* accession P95-99.1-1, only 4.7% of the 424 crosses made produced seed. Naskidashvili. (1984) reported no hybrid seed when he crossed seven different Georgian wheat cultivars, as the female parent, and *T. timopheevii*. He concluded that the failure was caused by hybrid lethality due to the interaction between the hybrid necrosis genes *Ne1* and *Ne2*. Peusha and Shnaider, (1983) crossed the wheat cultivar, Saratovskaya 29 as the female parent with *T. timopheevii* and only 5.4% of the 5704 florets pollinated set seed. Hence, genetic incompatibility, differences in genomic structure and the hybrid necrosis gene on 5A<sup>t</sup> (Tsunewaki, 1992) could all be the contributing factors for low hybrid seed production.

When attempting to transfer useful traits from *T. timopheevii* into wheat the different accessions of the wild parent varied in terms of crossability/fertility. In this study, six accessions of *T. timopheevii* ssp. *timopheevii* and ssp. *armeniicum* were used for crossing. The percentage of crosses producing F<sub>1</sub> seeds between wheat/*T. timopheevii* varied depending on the *T. timopheevii* accessions used. In accession P95-99.1-1, 4.7% of crosses produced seed but 62.5%, 21.4%, 100%, 75% and 100% of crosses produced seed for accessions 289752, 355452, 538512, 427414 and 427998, respectively (Table 3.5 and 3.6). However, it should be noted that a much lower number of crosses were carried out with the latter 5 accessions. This might be explained by: 1) The different glasshouses conditions when crossing with P95-99.1-1 the glasshouse used had been in continuous use for several crossing seasons and it has been noted that if a glasshouse was emptied after a crossing season and thoroughly cleaned and disinfected, plants were healthier. All other accessions were also crossed in a different glasshouse during a different year. Hence, although the same conditions were maintained as far as possible these might also have varied. 2) It could be possible that accession P95-99.1-1 is less crossable (may be due to translocations present, see Chapter 4) with wheat than the other five accessions (translocations unknown) leading to fewer crosses producing seeds.

All F<sub>1</sub> seed germinated were male sterile and were therefore backcrossed to wheat as female parents. This was supported by Jarve *et al.*, (2002), who stated that the self sterile F<sub>1</sub> hybrids between wheat and *T. timopheevii* can only be maintained by backcrossing them as the female with *T. aestivum*, with self-fertility usually

restored after 2-3 backcrosses. The fertility seen in the F<sub>1</sub> crossed ears (16.2%) improved in the later backcross generations, BC<sub>1</sub> (93.6%), BC<sub>2</sub> (92.5%), BC<sub>3</sub> (98.4%) and BC<sub>4</sub> (100%) respectively when crossed with wheat. The improvement in fertility was due to: 1) the gradual restoration of the normal chromosome number of 42 in the higher backcross generations. This in turn would lead to a more normal meiosis. This was also supported by van Slageren, (1994) where he proposed that sterility of most hybrids can be overcome by pollinating with the Triticum parent, restoring fertility and leading to further backcross generations that increasingly resemble wheat. 2) A reduction in the number of introgressions of *T. timopheevii* present in each of the genotypes.

The production of the BC<sub>1</sub> generation from the F<sub>1</sub> interspecific hybrids was not easy; out of 90 BC<sub>1</sub> individuals germinated only 35 plants grew to maturity and set seed. Thus, the total number of introgressions that could be generated would be limited to the 35 female F<sub>1</sub> gametes that gave rise to these 35 BC<sub>1</sub> plants if no further recombination occurred in the later generations.

The lines containing whole chromosome additions, substitutions and introgressed chromosome segments developed with *T. timopheevii* using the whole genome transfer approach, will not at this stage be very suitable for commercial exploitation due to the instability of the genomes i.e. the lines are heterozygous for the *T. timopheevii* introgressions and therefore are still segregating and also due to linkage drag. Lines have to be developed that contain only single introgressions and these lines then need to be stabilised, i.e. made homozygous, for future work. Once the lines are made homozygous, they will then be distributed for phenotyping for

target traits. Selfing of many of the lines to make them homozygous has been initiated. Markers are also currently being developed (see chapter 4) to enable detection of homozygous lines.

## **Chapter 4**

### **Characterization of the introgressions using molecular markers and *in situ* hybridization**

#### **4.1 Introduction**

The main techniques used to detect chromosome introgressions from *T. timopheevii* in the wheat genome have been C-banding, genomic *in situ* hybridization (GISH) and fluorescence *in situ* hybridization (FISH) (Badaeva *et al.*, 1991, 2000, 2010; Jiang and Gill, 1994a; Rodriquez *et al.*, 2000a; Gordeeva *et al.*, 2009; Miko *et al.*, 2015). Although C-banding was the first technique used to identify *T. timopheevii* chromosomes in the wheat genome, the more recent microscope-based techniques, GISH or mcGISH and FISH can be used to detect the introgressions and translocation breakpoints efficiently and complement classical diagnostic and selection tools. However, screening large numbers of plants or introgression lines is very difficult as these techniques are both time-consuming and technically demanding. In addition, minor rearrangements, or the rearrangement of chromosomal regions without a characteristic FISH pattern, cannot be identified by FISH. The availability of molecular markers makes it possible to overcome such difficulties. So, the GISH and FISH methods can be complemented with molecular markers methods (Uhrin *et al.*, 2012; Timonova *et al.*, 2013).

A number of molecular markers such as RFLP (restriction fragment length polymorphism), AFLP (amplified fragment length polymorphism), ESTs (expressed sequence tags), STS (sequence tagged site), COS (conserved orthologous set) and SSR (simple sequence repeats/microsatellites) have been exploited both in the past and in more recent studies involving *T. timopheevii*, for the identification, tagging

and mapping of chromosome segments mainly carrying disease resistance genes. Leonova *et al.* (2002, 2004, 2007, 2011) used SSR markers to map the leaf rust resistance genes and QTL on chromosome 2A (*LrTt1*), 2B (*QLr.osu-2B*) found later to be located in the same chromosome region as *Lr50* (Brown-Guedira *et al.*, 2003) and 5B (*LrTt2*) which was assumed to be same gene or a novel allele of *Lr18* (Friebe *et al.*, 1996), respectively. Wu *et al.* (2009) identified microsatellite marker loci *Xwmc344*, *Xwmc474* and *Xgwm374* closely linked to *Sr40* in the RL6088/Lakin linkage map. Bariana *et al.* (2001) identified 10 molecular markers (8RFLP and AFLP and 2SSRs) linked to the *Sr36* locus. Because these primers were not suitable to distinguish genotypes that were heterozygous from ones that were homozygous for *Sr36*, Tsilo *et al.* (2008) identified and validated a further three microsatellite markers (*Xgwm319*, *Xstm773-2* and *Xwmc477*) for the detection of *Sr36* in wheat breeding programs. Qin *et al.* (2011) studied the *Pm6* gene and mapped 28 markers including STS markers, conserved markers (based on Triticeae ESTs) and COS markers to a region on chromosome 2BL, of which 14 co-segregated with *Pm6*. The dominant powdery mildew resistance gene *Pm27*, was mapped to a 6B-6G translocation chromosome using RFLP and SSR markers and the gene was shown to co-segregate with the microsatellite locus *Xpsp3131* (Jarve *et al.*, 2000; Priilinn *et al.*, 2002). Srnic *et al.* (2005) used AFLP and SSR markers and reported the SSR markers *Xgwm332* and *Xwmc525* as flanking the powdery mildew resistance gene *Pm37* with genetic distances of 2.0cM and 1.4cM respectively on chromosome arm 7AL. *Pm37* was found to be located 16cM from another powdery mildew resistance gene *Pm1* (Perugini *et al.*, 2008; Schneider *et al.*, 1991; Singrun *et al.*, 2004). One more dominant resistance gene (*MIAG12*) was mapped on

chromosome arm 7AL which was flanked by SSR markers *Xwmc346* and *Xwmc273* (Maxwell *et al.*, 2009). Recently, a new leaf rust resistance gene (*LrSelG12*) was mapped to chromosome 3BL with the SSR markers *Xgwm114* and *Xgwm547* (Singh *et al.*, 2017). The *Br-At1* (brittle rachis) gene was mapped in a recombinant inbred line population of *T. araraticum* - *T. timopheevii* using SSR markers, to a 7.4cM region of the chromosome arm 3A<sup>t</sup>S, the region being flanked by markers *Xgwm2* and *Xbem44* (Rawat *et al.*, 2012).

Mapping of wheat microsatellite loci on *T. timopheevii* chromosomes by comparative mapping using microsatellites derived from bread wheat, led to the construction of genetic maps for two crosses: *T. timopheevii* var. *timopheevii* X *T. timopheevii* var. *typica* and *T. timopheevii* K-38555 X *T. militinae* (Salina *et al.*, 2006). The constructed maps were further compared with the International Triticeae Mapping Initiative (ITMI) population of *T. aestivum* (Roder *et al.*, 1998). The transferability of the wheat markers to *T. timopheevii* was generally better for the A genome-specific markers than the B and D genome-specific markers. The number of transferable wheat EST-SSR markers in the *T. timopheevii* genome was significantly higher than that of SSR markers (Dobrovolskaya *et al.*, 2009) and both were used in the construction of genetic maps of the A<sup>t</sup> genome of *T. timopheevii* and to study the synteny between the A<sup>t</sup> genome of *T. timopheevii* and the A genome of wheat (Dobrovolskaya *et al.*, 2009). Wheat SSR markers were largely exploited in the past and allowed the use of these markers for integrating target *T. timopheevii* genes or loci into the wheat genome or mapping resistance genes and



for detecting introgressions from *T. timopheevii* into wheat genotypes (Leonova *et al.*, 2002, 2008; Gordeeva *et al.*, 2009; Timonova *et al.*, 2012a, b, 2013).

Although there has been considerable use of *T. timopheevii* for the transfer of disease resistance genes only a few have been successful for wheat breeding. Among all the disease resistance genes transferred from *T. timopheevii* only the *Sr36* and *Pm6* genes have been broadly used in commercial wheat breeding (McIntosh *et al.*, 2013). The major limitation to successful transfers from *T. timopheevii* has been the lack of a high-throughput screening technology to identify and characterize introgressions, as the markers available until now did not provide a high enough resolution and were relatively low throughput. However, the development of next-generation sequencing technologies and single nucleotide polymorphism (SNP) markers, used in the current study provide a mechanism for the detection of introgressions into wheat from wild relatives as was demonstrated for *Ae. geniculata* (Tiwari *et al.*, 2014) and *Am. muticum* (King *et al.*, 2017).

#### **4.1.1 Aims and Objectives of this chapter**

The main aims of this chapter are:

- 1) The detection and characterization of *T. timopheevii* introgressions into wheat via wheat/wild relative SNP array (Axiom 35K genotyping array).
- 2) Validation of the genotyping by fluorescence *in situ* hybridisation.
- 3) Comparison of the syntenic relationship of *T. aestivum* with *T. timopheevii*

## **4.2 Materials and Methods**

### **4.2.1 Plant Material**

The individuals used for genotyping (Table 1, Appendix) were generated from the cross made between *T. timopheevii* ssp. *timopheevii*, accession P95-99.1-1 and wheat cultivar (var. Paragon) as shown in Chapter 3, (section 3.2.1).

### **4.2.2 DNA extraction**

Harvesting of leaf tissue, genomic DNA extraction and quantification and preparation of the DNA samples for use in the Affymetrix axiom array (Affy) was described in Chapter 2, (section 2.4 and 2.6.3). The DNA was sent to the University of Bristol, for genotyping (see below).

### **4.2.3 Genotyping methodology**

The Axiom® Wheat-Relative genotyping Array was run at the University of Bristol on the Affymetrix Gene Titan® 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 powertools (APT) and SNPolisher™ ([http://www.affymetrix.com/estore/partners\\_programs/programs/developer/tools/devnettools.affx](http://www.affymetrix.com/estore/partners_programs/programs/developer/tools/devnettools.affx)). A custom software pipeline ADAP (Axiom® Data Analysis Pipeline) was written in Perl (<http://www.perl.org>) to simplify the data analysis, following the Axiom® Best practices genotyping Workflow ([http://media.affymetrix.com/support/downloads/manuals/axiom\\_genotyping\\_solution\\_analysis\\_guide.pdf](http://media.affymetrix.com/support/downloads/manuals/axiom_genotyping_solution_analysis_guide.pdf)). A variant call rate threshold of 80% was used instead of the default value (97%) to account for the lower call rates typically obtained from hybridising wheat relatives and progenitors to the array. The apt-probeset-

genotype programme within Affymetrix Power Tools was used to determine the genotype calls from the Affymetrix SNP arrays. The SNPolisher R package was then used to calculate SNP performance metrics, such as call rate, cluster separation and deviation from expected cluster position before classifying the SNPs into performance categories. These categories were: 1) Polymorphic high resolution (PHR) calls which were considered to give good cluster resolution with at least two examples of the minor alleles, 2) Monomorphic high resolution (MHR) calls with less than two examples of the minor allele, 3) off-target variant, where variants were reproducible and interfere with the SNP of interest, creating a fourth cluster, 4) No minor homozygous, where two clusters with no example of the minor homozygous genotypes, 5) Call rate below threshold (CRBT), where sample call rate was below threshold but other cluster properties were above threshold, 6) Other, where one or more cluster properties were below threshold. PHR calls were the only calls used in genotyping as these were considered to represent good cluster resolution and hence were the most accurate.

The underlying principal is the specific hybridization of DNA fragments from a test sample to complementary sequences on the microarray (Kennedy *et al.*, 2003). The array consists of 1X 384 sample plates, i.e. 384 samples can be analyzed on the one array. The analysis was done with AxiomGT1 with generic priors with 36,712 wheat-relative probesets.

#### **4.2.4 Genetic mapping of *T. timopheevii***

The Axiom<sup>®</sup> wheat-Relative Genotyping Array (hereafter referred to as the Axiom<sup>®</sup> array) was used to screen genomic DNA prepared from individuals from a backcross population between *T. aestivum* and *T. timopheevii*. Samples analysed included the

wheat and *T. timopheevii* (P95-99.1-1), parental lines and 504 introgression lines comprising BC<sub>1</sub>, BC<sub>2</sub>, BC<sub>2</sub>F<sub>1</sub>, BC<sub>2</sub>F<sub>2</sub>, BC<sub>3</sub>, BC<sub>3</sub>F<sub>1</sub> and BC<sub>4</sub> populations (Appendix 2). For genetic mapping purposes, only the PHR SNP markers were used as they provide good cluster resolution where each SNP essentially behaves like a diploid. Flapjack™ (Milne *et al.*, 2010; v.1-14.09.24) software was used to delete all the SNP markers which showed heterozygous calls of either parent, no polymorphism between the wheat parents and *T. timopheevii* and no calls for either parent. JoinMap® 4.1 (van Ooijen, 2011) was then used to sort all the resulting markers into linkage groups with a LOD (Log-odds) score of 30 for some linkage groups and 60 for others and a recombination frequency threshold of 0.1 using the Haldane mapping function (Haldane, 1919). The highest ranking linkage groups with more than 30 markers were selected for map construction. The ordered groups were exported and assigned to chromosomes using information from the Axiom® Wheat HD Genotyping Array (Winfield *et al.*, 2016). If the chromosomes were split into multiple linkage groups, they were re-formed into a single linkage group and again re-ordered using JoinMap. Markers that shared their pattern of segregation with other markers were retained. Markers with more than 20% missing data points were removed. Markers which were present in advanced backcross progeny but absent in earlier backcross generations and/or not consistent with adjacent marker in the group were also removed. The IWGSC wheat survey sequence (The International wheat Genome sequence Consortium, 2014) was used to identify the chromosome arms i.e. long and the short arms. Once the chromosome arms were identified, the groups were oriented to have the short arm above the long arm. Reordering of the markers was done according to the marker order in the short arm

and long arm as compared to wheat in a best possible fit across all families, while maintaining them in the correct arm. The final genetic maps were then produced through MapChart 2.3 (Voorrips, 2002). Graphical Genotypes 2.0 (GGT; van Beeloo, 2008) was used to perform the visualization to track the introgressions.

#### **4.2.5 *In situ* hybridization**

##### **4.2.5.1 FISH**

Fluorescence *in situ* hybridization (FISH) was carried out on metaphase spreads of introgression lines with probes based on the cloned repetitive DNA sequences- Afa-family, pSc119.2. For three-colour FISH, the pSc119.2 and Afa-family DNA sequences were amplified and labeled by PCR with biotin-11-dUTP and digoxigenin-16-dUTP, respectively. The methodology for making the probes, their PCR amplification and, probing of the slides has been given in Chapter 2 (section 2.5.7). Fluorescent signals were visualized with a Leica DM5500B epifluorescence microscope (Leica Microsystems, Wetzlar, Germany) with a filter for detecting DAPI and a dual band filter set to observe FITC and rhodamine signals simultaneously i.e. DAPI (blue), Alexa Fluor 488 (green) and Alexa Fluor 594 (red). Photographs were taken with a Leica DFC 350FX digital camera.

##### **4.2.5.2 FISH followed by Multicolour GISH (mcGish)**

For detection of the intergenomic translocations present in *T. timopheevii*, (accession P95-99.1-1), FISH as described above on a metaphase spread of *T. timopheevii* was followed by mcGISH. After photographing the slide of FISH, the slides were washed (method described in Chapter 2, section 2.5.6) and rehybridised and GISH was carried out as discussed in Chapter 2, section 2.5.6. For GISH, total genomic DNA of *T. urartu* and *Ae. speltoides* were used as probes. The

slides were counterstained with DAPI. Analysis and visualization were as for FISH (section 4.2.6.1).

## **4.2.6 Development of KASP markers**

### **4.2.6.1 Primer design**

All the SNP markers from the Axiom® Wheat-Relative 35K Array were put into a blast search to *Triticum aestivum* on the URGI website (<http://urgi.versailles.inra.fr/blast/blast.php>) to find the IWGC codes for each SNP. Using the IWGC codes, the SNP markers were assigned to their specific chromosome arm. I was assigned group 3 and group 6.

The sequence of each marker was put into a Blast search in *EnsemblPlants* ([http://plants.ensembl.org/Triticum\\_aestivum/Info/Index](http://plants.ensembl.org/Triticum_aestivum/Info/Index)) against *Triticum aestivum* TGACv1 (Genomic sequence). The scaffold positions with the top hit and a score above 90 were selected. The genomic location, i.e. the position of the marker, the overlapping genes to that marker and the genetic bin wherever available, were then noted for each marker. The selected alignment was also checked to insure that the selected alignment was between exons and not at the end of the sequence. The query sequence was then used for primer design via the primer3 (Primer3web version 4.0.0) input website (<http://primer3.ut.ee/>), where the product size range was set to 500-550 base pairs. These steps were followed for each SNP marker and thus primers were designed for each marker.

### **4.2.6.2 Sequence of markers**

A total of 2,169 markers were designed for all seven chromosomes which included 261 primers for group 1, 334 primers for group 2, 428 primers for group 3, 214

primers for group 4, 287 primers for group 5, 309 primers for group 6 and 336 primers for group 7, respectively.

#### 4.2.6.3 SNP Discovery

The primer designed above were used to PCR amplify DNA from two of the parental wheat cultivars (Chinese Spring and Paragon) plus the 10 wild relatives given in Chapter 2 (section 2.6.1). The PCR mixture and profile are given below (Table 4.1 and 4.2). All PCR reactions were carried out using an Eppendorf Master Cycler (Applied Biosystems gene Amp 9700).

**Table 4.1:** Final concentration of different components for PCR reaction for SNP design

| Components                         | Volume ( $\mu$ l) |
|------------------------------------|-------------------|
| Genomic DNA( $\sim$ 50ng/ $\mu$ l) | 5                 |
| ddH <sub>2</sub> O                 | 16.18             |
| 10x Buffer with Mg++               | 2.5               |
| dNTP's mix (10 $\mu$ m each)       | 0.125             |
| Forward Primer (10 $\mu$ m)        | 0.5               |
| Reverse primer (10 $\mu$ m)        | 0.5               |
| Taq polymerase                     | 0.2               |
| Total                              | 25                |

**Table 4.2:** Temperature profile used for PCR for SNP design

| Step                 | Temperature(°C) | Time (minutes) | No. of cycles |
|----------------------|-----------------|----------------|---------------|
| Initial denaturation | 95.0            | 05:00          |               |
| Denaturation         | 94.0            | 00:30          | x10           |
| Annealing            | 65.0*           | 00:30          |               |
| Elongation           | 72.0            | 01:00          |               |
|                      | 94.0            | 00:30          | x28           |
|                      | 58.0            | 00:30          |               |
|                      | 72.0            | 01:00          |               |
| Final Extension      | 72.0            | 05:00          |               |
| Hold                 | 18.0            |                |               |

#### 4.2.6.4 Visualization of PCR products

The PCR products were run on a 1.5% agarose electrophoresis gel (Bioline, superfine resolution, Molecular grade) made in 1X TAE buffer (Appendix 1). 3.0µl of 6X loading dye was added to 25 µl of the amplified product. Ethidium bromide was added at a concentration of 0.5 µg/µl. 5µl of 1Kb ladder was also run. The gel was run at 190v for 1-1.5h, visualised under UV light and photographed using UVP gel documentation system (Syngene, Scientific Laboratory Supplies).

#### 4.2.6.5 DNA purification

Following electrophoresis, where bands of the correct size had been amplified from the DNA of the wild relatives, these were cut from the agarose gel and purified using the NucleoSpin® Gel and PCR Clean-up Kit following the manufacturer's instructions:

500µl of NT1 buffer was added to the 1.5 ml eppendorf tubes containing the gel slices. The tubes was incubated for 5-10 min at 50°C in the heating block with the



samples vortexed briefly every 2-3 mins until the gel slices was completely dissolved.

NucleoSpin® Gel and PCR clean-up Column was placed into a collection tube and a total of 700 µl of the sample was loaded into it. The tubes were centrifuged for 30 seconds at 11,000 x *g*. The supernatant was discarded and the column was placed back into the collection tube. Any remaining sample was loaded and the centrifugation step was repeated.

700 µl of Buffer NT3 was added to the NucleoSpin® Gel and PCR Clean-up Column. The tubes was then centrifuged for 30 seconds at 11,000 x *g* and the supernatant discarded and the column placed back into the collection tube. This washing step was done twice.

The tubes were centrifuged for a further 1 min at 11,000 x *g* to ensure all NT3 buffer was completely removed. After centrifugation, the tubes were left to air dry for 1 minute.

The NucleoSpin® Gel and PCR Clean-up Column were placed into a clean 1.5 mL microcentrifuge tube and 16 µl of ddh<sub>2</sub>O was added. The tubes were then centrifuged for 1 minute at 11,000 x *g* and the supernatant collected.

#### **4.2.6.6 Sequencing**

The purified samples were sent to Source BioScience (<http://www.sourcebioscience.com/>) for Sanger sequencing. For each sequencing reaction 5µl of both primer (3.2 pmol/µl) and DNA (1 ng/µl per 100 base pair) was sent. The sequencing templates were found to be G-C rich, contained hairpin structures and repetitive regions and therefore the Source Bioscience Secondary

structure treatment solution (dGTP chemistry) was performed along with the sequencing.

#### **4.2.6.7 Sequencing Analysis**

All the sequencing results were analysed using Chromas lite (version 2.6.2) to find the quality of the sequences. Only the sequences which had big peaks and good quality were used for the analysis. The sequences which had double peaks and no clear reads were discarded. In some cases, if 100 bp of the sequence was good quality and rest was double peak, still the sequences were taken and analysed.

Genedoc (version 2.7) was used to align the good quality sequence for a given marker. Following the pairwise alignment of the sequences, if big region of insertion or deletion was found, the sequence was checked again for the quality. If the sequence quality was good it was expected to have deletion or insertion at the sequence site and sequence for that species was deleted from the pairwise alignment analysis. Gap columns created in the sequences were removed. Usually a common SNP between most of the species was looked for designing the SNP. Once the SNP is identified, it was highlighted and replaced with the IUPAC code (IUPAC SNP nomenclature) with square brackets for the target SNP in Chinese Spring sequence. A region was marked along either sides of the target SNP (usually 50-60bp) and all the SNPs lying in the target region were replaced with the IUPAC code but without square brackets. The region around target SNP was selected in such a way that not more than 4 to 5 SNPs were present either side of target SNP.

To verify the chromosome arm for particular marker the sequence of each marker was put into a Blast search in *EnsemblPlants* ([http://plants.ensembl.org/Triticum\\_aestivum/Info/Index](http://plants.ensembl.org/Triticum_aestivum/Info/Index)) against *Triticum*

*aestivum* TGACv1 (Genome sequence) and also in NRGene assembly, Sequence server website (10.157.8.165:4567), advanced parameter (-evalue 1.0e-10-max\_target\_seq\_3) where every chromosome in wheat got a physical position for every base. In each case, the scaffold positions with the top hit having the score above 90 were looked up for any other copies of the sequence in other chromosomes or same chromosome but other arm. The presence of the copy of the target SNP in the same chromosome but other arm was noted down for the SNP marker. The Popseq position from the Sequence server website for the target SNP, the chromosome arm where it is located, the species allele and the wheat allele, the SNP IUPAC code, the SNP position in Genedoc and the sequence of the target SNP were recorded for each marker. Table 4.3 shows the number of primers designed and amplified and the SNP identification for all the species and in particular *T. timopheevii* for chromosome 3 and 6, respectively.

**Table 4.3:** Total number of primers designed and giving amplification products, SNP identification for all 10 wild species and in particular for *T. timopheevii* for chromosome 3 and 6.

| Chromosome | No. of Primer designed | Primer amplifying | SNP identification (all species) | SNP identification ( <i>T. timopheevii</i> ) |
|------------|------------------------|-------------------|----------------------------------|----------------------------------------------|
| 3A         | 86                     | 79                | 50                               | 29                                           |
| 3B         | 266                    | 194               | 118                              | 33                                           |
| 3D         | 76                     | 61                | 36                               | 6                                            |
| 6A         | 97                     | 83                | 55                               | 11                                           |
| 6B         | 84                     | 72                | 45                               | 12                                           |
| 6D         | 128                    | 111               | 66                               | 7                                            |

The sequences containing SNPs are being used for KASP design. The design of the KASP markers and their validation is being carried out by LGC. Validation of the KASP markers for *T. timopheevii* is being carried out on 1106 genotypes including

59 BC<sub>2</sub>, 364 BC<sub>2</sub>F<sub>1</sub>, 173 BC<sub>3</sub>, 233 BC<sub>3</sub>F<sub>1</sub>, 102 BC<sub>3</sub>F<sub>2</sub>, 124 BC<sub>4</sub> and 51 BC<sub>4</sub>F<sub>1</sub>. This analysis is due for completion in November 2017.

## 4.3 Results

### 4.3.1 Genetic mapping of *T. timopheevii*

The individuals used for genotyping included parental lines (Paragon, Chinese spring, Pavon, Highbury and *T. timopheevii*, accession P95-99.1-1) and 504 introgression lines comprising 35 BC<sub>1</sub>, 134 BC<sub>2</sub>, 27 BC<sub>2</sub>F<sub>1</sub>, 57 BC<sub>2</sub>F<sub>2</sub>, 145 BC<sub>3</sub>, 73 BC<sub>3</sub>F<sub>1</sub> and 33 BC<sub>4</sub> populations, respectively.

After genotyping with the axiom array, 2844 SNP markers were polymorphic between *T. timopheevii* and wheat (var. Paragon). The PHR SNPs used in map construction using JoinMap® (van Ooijen, 2011), resulted in fourteen linkage groups of *T. timopheevii*.

In total, 1531 SNP markers were mapped in the population across all 14 chromosome of *T. timopheevii* (Figure 4.1 and Figure 4.2). The number of markers per chromosome varied, with some linkage groups with higher numbers of markers than others, e.g. 5G (218), 6G (176), 7G (161), 1G (155), 2G (146), 3G (133), 4G (123) and 4A<sup>t</sup> (36), 5A<sup>t</sup> (44), 6A<sup>t</sup> (54), 1A<sup>t</sup> (65), 7A<sup>t</sup> (65), 2A<sup>t</sup> (70), 3A<sup>t</sup> (85), respectively (Table 4.4). The cM lengths of fourteen linkage groups were 45.77 for 1A<sup>t</sup>, 19.225 (1G), 66.129 (2A<sup>t</sup>), 188.32 (2G), 17.23 (3A<sup>t</sup>), 9.365 (3G), 16.063 (4A<sup>t</sup>), 22.905 (4G), 72.715 (5A<sup>t</sup>), 48.181 (5G), 49.551 (6A<sup>t</sup>), 44.62 (6G), 52.08 (7A<sup>t</sup>) and 22.918 (7G), respectively. Hence, the total map length was 675.05 cM with an average chromosome length of 48.219 cM. No regular patterns in distribution of SNP polymorphic markers over the fourteen chromosomes but were generally well distributed across all the linkage groups of *T. timopheevii*.

**Figure 4.1:** Genetic linkage map of A<sup>t</sup> genome of *T. timopheevii*. SNP marker names and cM distances for each of the linkage group are given in Appendix 3.

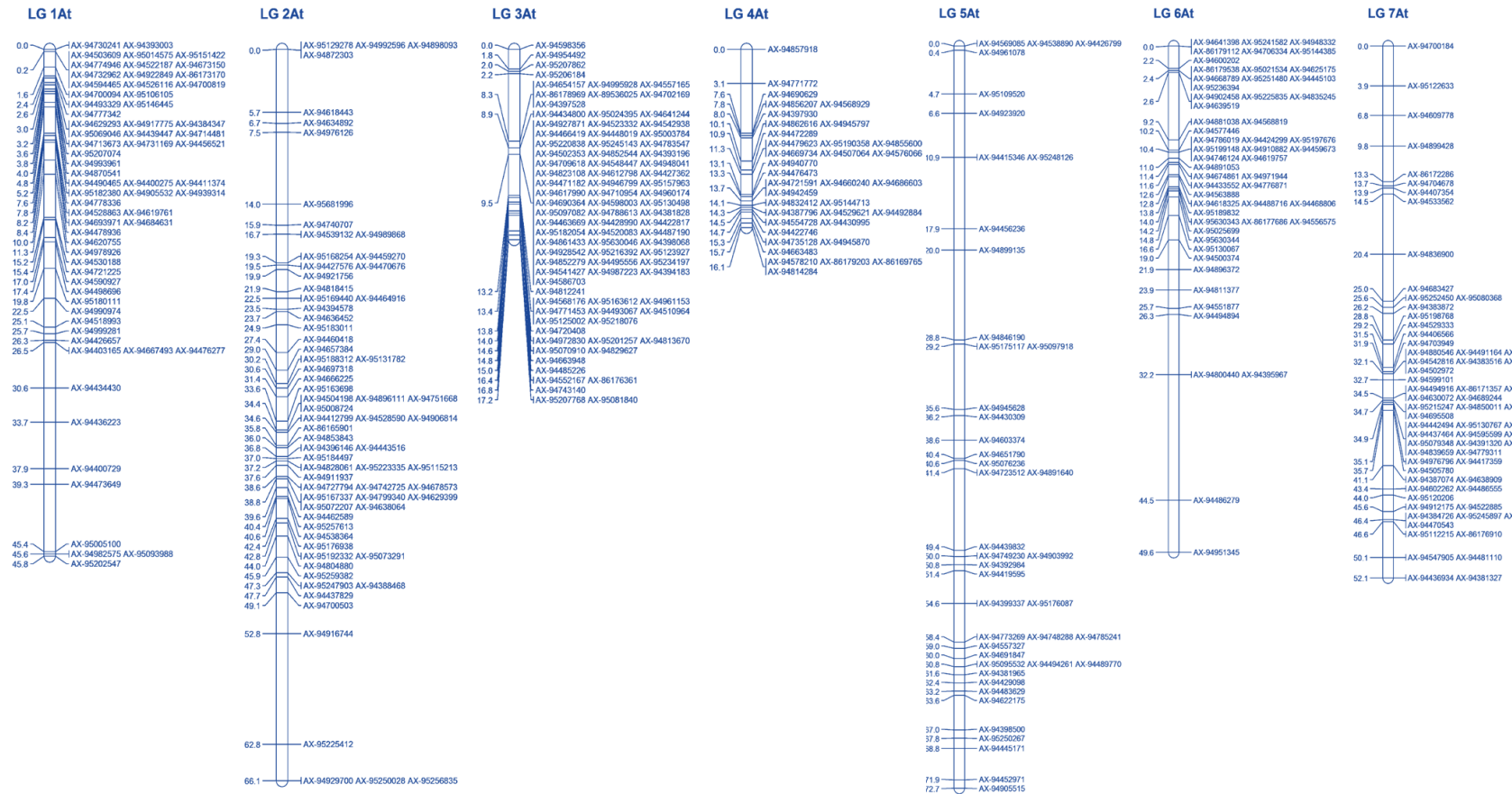


**Figure 4.2:** Genetic linkage map of G genome of *T. timopheevii*. SNP marker names and cM distances for each of the linkage group are given in Appendix 3.

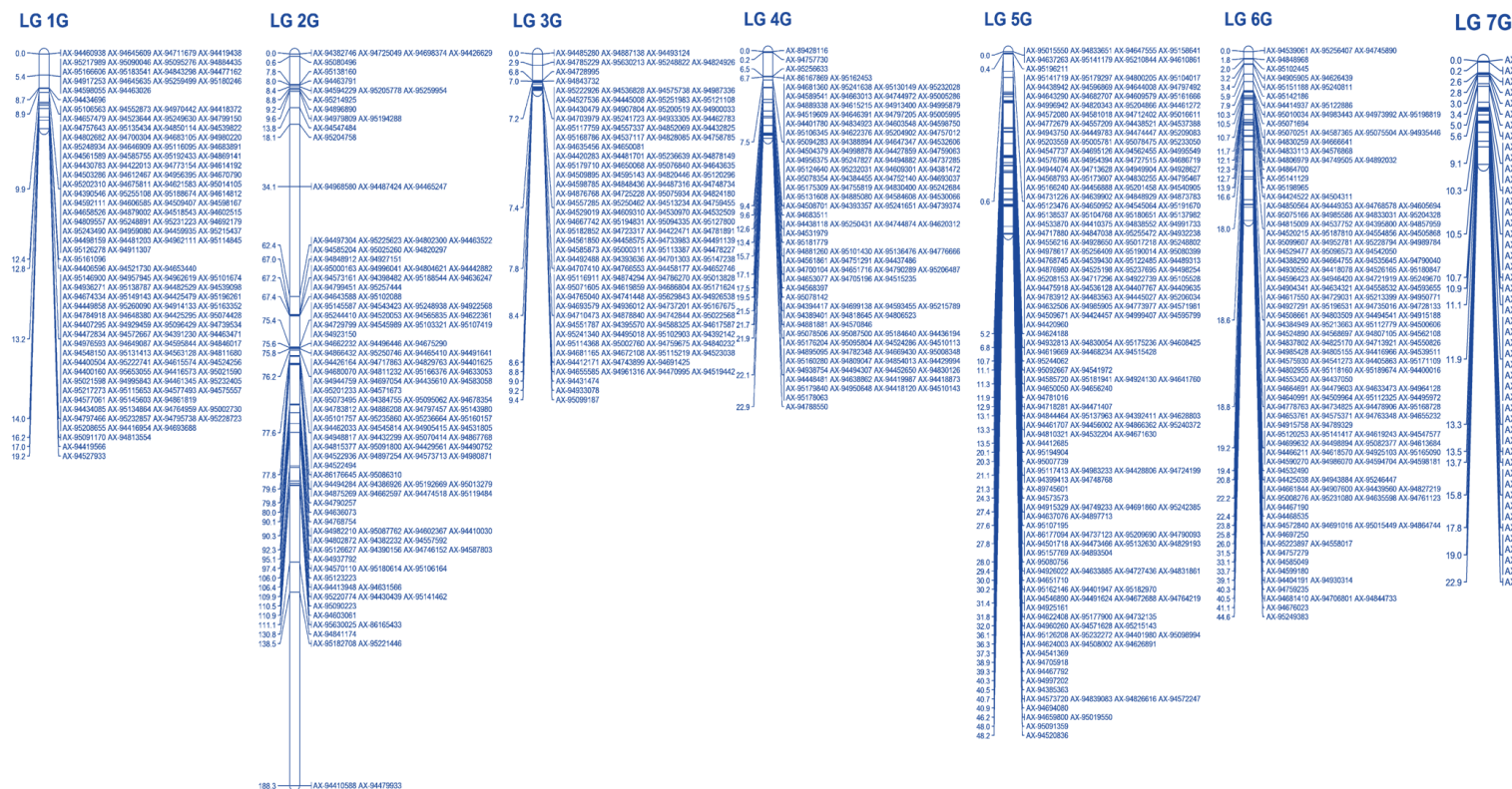


**Table 4.4:** Total number of SNP (single nucleotide polymorphic markers) mapped on genetic linkage map of A<sup>t</sup> and G genome of *T. timopheevii* and their respective lengths (cM).

| S. No.       | Linkage groups    | No. of SNPs  | Length (cM) |
|--------------|-------------------|--------------|-------------|
| 1            | LG1A <sup>t</sup> | 65           | 45.77       |
| 2            | LG1G              | 155          | 19.225      |
| 3            | LG2A <sup>t</sup> | 70           | 66.129      |
| 4            | LG2G              | 146          | 188.323     |
| 5            | LG3A <sup>t</sup> | 85           | 17.23       |
| 6            | LG3G              | 133          | 9.365       |
| 7            | LG4A <sup>t</sup> | 36           | 16.063      |
| 8            | LG4G              | 123          | 22.905      |
| 9            | LG5A <sup>t</sup> | 44           | 72.715      |
| 10           | LG5G              | 218          | 48.181      |
| 11           | LG6A <sup>t</sup> | 54           | 49.551      |
| 12           | LG6G              | 176          | 44.62       |
| 13           | LG7A <sup>t</sup> | 65           | 52.08       |
| 14           | LG7G              | 161          | 22.918      |
| <b>Total</b> | <b>14</b>         | <b>1,531</b> | 675.075     |



Genetic mapping of the SNP markers revealed that a total of 1,807 introgressions were generated between *T. timopheevii* and wheat, which covered the entire genome of *T. timopheevii*. The chromosomes containing introgressions were divided into two groups. The first group included chromosomes 1A<sup>t</sup>, 2G, 3A<sup>t</sup>, 4G, 5A<sup>t</sup>, 6G and 7A<sup>t</sup> in which introgressions occurred with high frequency. The second group included chromosomes 1G, 2A<sup>t</sup>, 3G, 4A<sup>t</sup>, 5G, 6A<sup>t</sup> and 7G with lower introgression levels (Table 4.5). On the basis of the data obtained, it can be concluded that the number of introgression fragment retained decreased with the number of backcrosses with respect to all the fourteen linkage groups of *T. timopheevii*. For instance, for LG1A<sup>t</sup>, 68.57% of BC<sub>1</sub> genotypes contained introgressions which decreased to 48.50% in the BC<sub>2</sub>, 28.96% in the BC<sub>3</sub> and only 15.15% in the BC<sub>4</sub>. For LG2A<sup>t</sup> 80% of BC<sub>1</sub> genotypes contained introgressions which reduced to 48.50% in BC<sub>2</sub> and 32.41% in BC<sub>3</sub> and 9.09% in BC<sub>4</sub> (Table 4.5). The only exception to this observation was chromosome 2G where 94.28% of BC<sub>1</sub> genotypes contained introgressions but this reduced only slightly in further generations to 80.59% in the BC<sub>2</sub>, 75.17% in the BC<sub>3</sub> and 72.72% in the BC<sub>4</sub>. The preferential inheritance of the 2G chromosome and its fragments in the backcross generations led to the highest number of introgressions (274) out of all the chromosomes.

**Table 4.5:** Frequency of introgressions in hybrids between *T. aestivum* and *T. timopheevii* for Linkage group 1 (LG1), Linkage group 2 (LG2), Linkage group 3 (LG3), Linkage group 4 (LG4), Linkage group 5 (LG5), Linkage group 6 (LG6) and Linkage group 7 (LG7) with respect to two genomes ( $A^t$  and G) of *T. timopheevii*.

| S. No. | Linkage group | Genomes        | 35 BC <sub>1</sub> plants (%) | 134 BC <sub>2</sub> plants (%) | 145 BC <sub>3</sub> plants (%) | 33 BC <sub>4</sub> plants (%) | Total |
|--------|---------------|----------------|-------------------------------|--------------------------------|--------------------------------|-------------------------------|-------|
| 1      | LG1           | A <sup>t</sup> | 24 (68.57%)                   | 65 (48.50%)                    | 40 (27.58%)                    | 5 (15.15%)                    | 134   |
| 2      |               | G              | 14 (40%)                      | 26 (19.4%)                     | 10 (6.89%)                     | -                             | 50    |
| 3      | LG2           | A <sup>t</sup> | 28 (80%)                      | 65 (48.50%)                    | 47 (32.41%)                    | 3 (9.09%)                     | 143   |
| 4      |               | G              | 33 (94.28%)                   | 108 (80.59%)                   | 109 (75.17%)                   | 24 (72.72%)                   | 274   |
| 5      | LG3           | A <sup>t</sup> | 22 (62.85%)                   | 48 (35.82%)                    | 35 (24.13%)                    | -                             | 105   |
| 6      |               | G              | 17 (48.57%)                   | 32 (23.88%)                    | 22 (15.17%)                    | -                             | 71    |
| 7      | LG4           | A <sup>t</sup> | 25 (71.42%)                   | 48 (35.82%)                    | 23 (15.86%)                    | 5 (15.15%)                    | 101   |
| 8      |               | G              | 24 (68.57%)                   | 51 (38.05%)                    | 24 (16.55%)                    | 6 (18.18%)                    | 105   |
| 9      | LG5           | A <sup>t</sup> | 33 (94.28%)                   | 82 (61.19%)                    | 56 (38.62%)                    | 8 (24.24%)                    | 179   |
| 10     |               | G              | 32 (91.42%)                   | 74 (55.22%)                    | 41 (28.27%)                    | 3 (9.09%)                     | 150   |
| 11     | LG6           | A <sup>t</sup> | 28 (80%)                      | 62 (46.26%)                    | 33 (22.75%)                    | 8 (24.24%)                    | 131   |
| 12     |               | G              | 27 (77.14%)                   | 57 (42.53%)                    | 44 (30.34%)                    | 9 (27.27%)                    | 137   |
| 13     | LG7           | A <sup>t</sup> | 28 (80%)                      | 68 (50.74%)                    | 47 (32.41%)                    | 6 (18.18%)                    | 149   |
| 14     |               | G              | 23 (65.71%)                   | 44 (32.83%)                    | 40 (27.58%)                    | 7 (21.21%)                    | 78    |
| Total  |               |                | 358                           | 830                            | 571                            | 84                            |       |

Out of all the backcross plants, all BC<sub>1</sub> plants had more than one segment present therefore it was the later generations where the segments were separating out. Molecular marker (SNP) analysis revealed, 28 plants with a single introgression from chromosomes 1A<sup>t</sup>, 2G, 3A<sup>t</sup>, 4G, 5A<sup>t</sup>, 5G, 6A<sup>t</sup>, 6G and 7A<sup>t</sup> of *T. timopheevii* and 64 plants each with two introgressions from the genome of the wild relative. None of the plants carried single introgressions involving either chromosome 1G, 2A<sup>t</sup>, 3G, 4A<sup>t</sup> and 7G. In the third backcross, the number of introgressed fragments in the progenies mostly ranged from one to four with no *T. timopheevii* genetic material detectable in one plant (BC<sub>3</sub>-100C). In the BC<sub>3</sub>F<sub>1</sub> plants, three introgressions was the most frequent number of introgressions i.e. present in 26 plants out of 73 BC<sub>3</sub>F<sub>1</sub>, while 5 plants (BC<sub>3</sub>F<sub>1</sub>-150C, 150E, 150G, 150H and 145D) showed no introgression present. In the fourth backcross, eight plants contained a single introgression, 7 plants contained 2 introgressions, 7 plants contained 3 introgression fragments and just one plant (BC<sub>4</sub>-132F) contained no introgression (Table 4.6).

**Table 4.6:** Different number of introgressions present in respective backcross (BC<sub>2</sub>, BC<sub>2</sub>F<sub>1</sub>, BC<sub>2</sub>F<sub>2</sub>, BC<sub>3</sub>, BC<sub>3</sub>F<sub>1</sub>, and BC<sub>4</sub>) generations.

| Backcross generation number    | Number of Introgressions |           |           |           |           |
|--------------------------------|--------------------------|-----------|-----------|-----------|-----------|
|                                | No                       | Single    | Two       | Three     | Four      |
| BC <sub>2</sub>                | 0                        | 0         | 2         | 8         | 19        |
| BC <sub>2</sub> F <sub>1</sub> | 0                        | 3         | 4         | 12        | 4         |
| BC <sub>2</sub> F <sub>2</sub> | 2                        | 4         | 14        | 13        | 12        |
| BC <sub>3</sub>                | 1                        | 5         | 26        | 27        | 37        |
| BC <sub>3</sub> F <sub>1</sub> | 5                        | 8         | 11        | 26        | 17        |
| BC <sub>4</sub>                | 1                        | 8         | 7         | 7         | 4         |
| <b>Total</b>                   | <b>9</b>                 | <b>28</b> | <b>64</b> | <b>93</b> | <b>93</b> |

#### 4.3.2 *In situ* hybridization

##### Karyotyping by FISH

Three-color FISH was carried out on the original parents in order to confirm that the *T. timopheevii* FISH karyotype published by Miko *et al.*, (2015) for the A<sup>t</sup> and G genomes held true for the accession used in the current crossing programme. Chromosome discrimination was carried out using the previously published FISH patterns of *T. timococcum* (synthetic amphidiploid between *T. timopheevii* and *T. monococcum*) (Miko *et al.*, 2015) and *T. aestivum* (Sepsi *et al.*, 2008). The chromosomes were identified by the distribution pattern of pSc119.2 and Afa-family blocks. The green colored pSc119.2 probe gave strong signals on all the G genome chromosomes and weaker signals on two A<sup>t</sup> chromosomes 1A<sup>t</sup> (interstitial band on the long arm) and 5A<sup>t</sup> (2 signals at the terminal end of the short arm). The Afa-family signals (red colour) when used in combination with pSc119.2, allowed the G genome chromosomes to be clearly distinguished from each other. The Afa-family

probe hybridized mainly to the chromosomes of A<sup>t</sup> genomes, which could be distinguished from the A genome of wheat using the reference karyotypes. Individual chromosomes were then cut out with the help of Image J software and assembled to show the karyotypes of the two genomes of *T. timopheevii* i.e. A<sup>t</sup> and G genomes (Figure 4.3). The three genomes of wheat/*T. aestivum* i.e. A, B and D genomes were also labeled on the metaphase spread of wheat (cv. Paragon) (Figure 4.4).

**Figure 4.3:** FISH with probe Afa-family (red signal) and pSc119.2 (green signal) on a) the metaphase spread of *T. timopheevii* ssp. *timopheevii*, accession P95-99.1-1 and b) karyotype of A<sup>t</sup> and G genomes: fluorescence *in situ* hybridization patterns of repetitive DNA probes pSc119.2, Afa-family on chromosomes of *T. timopheevii* ssp. *timopheevii*, accession P95-99.1-1 arranged according to genomes.

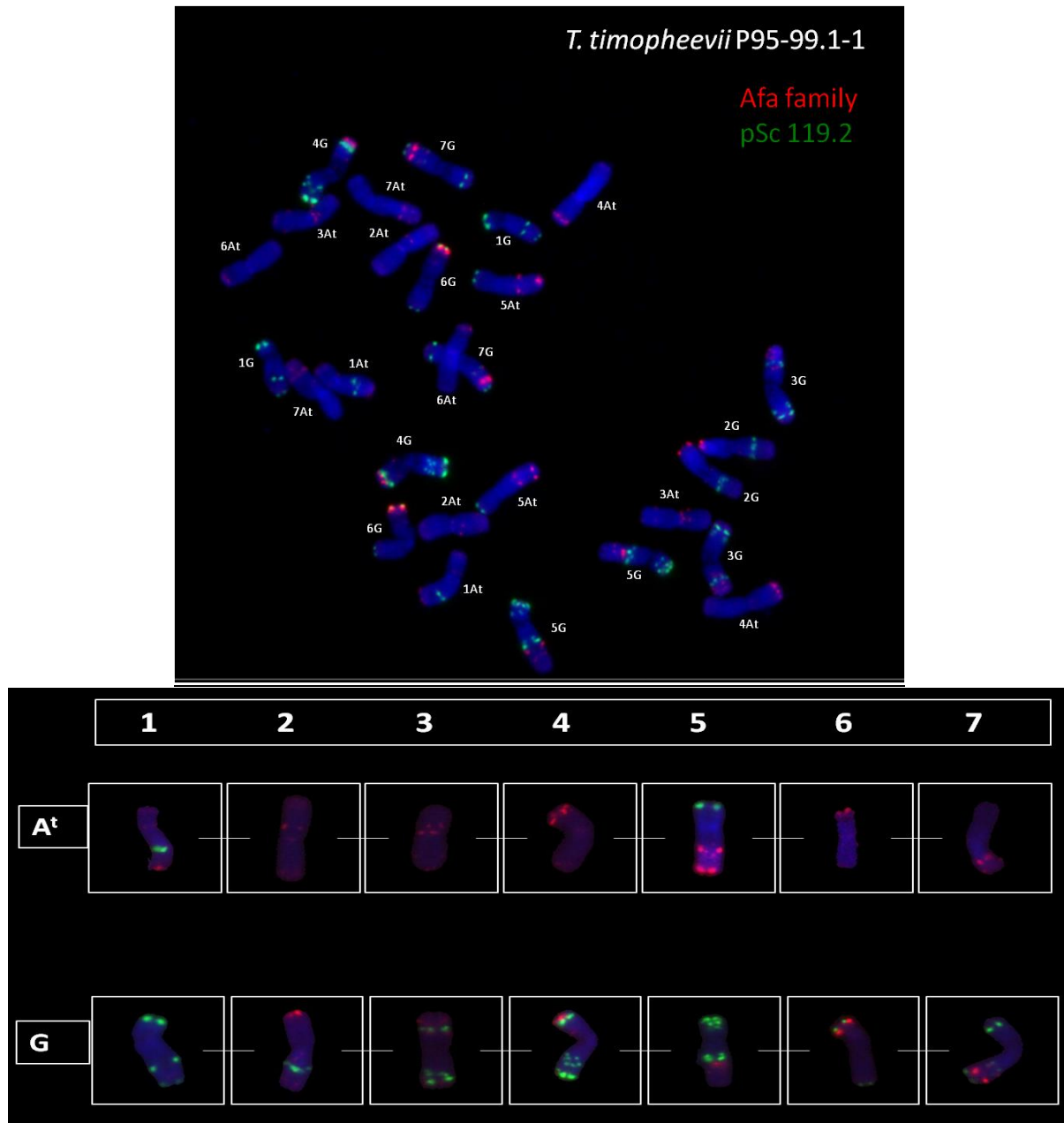
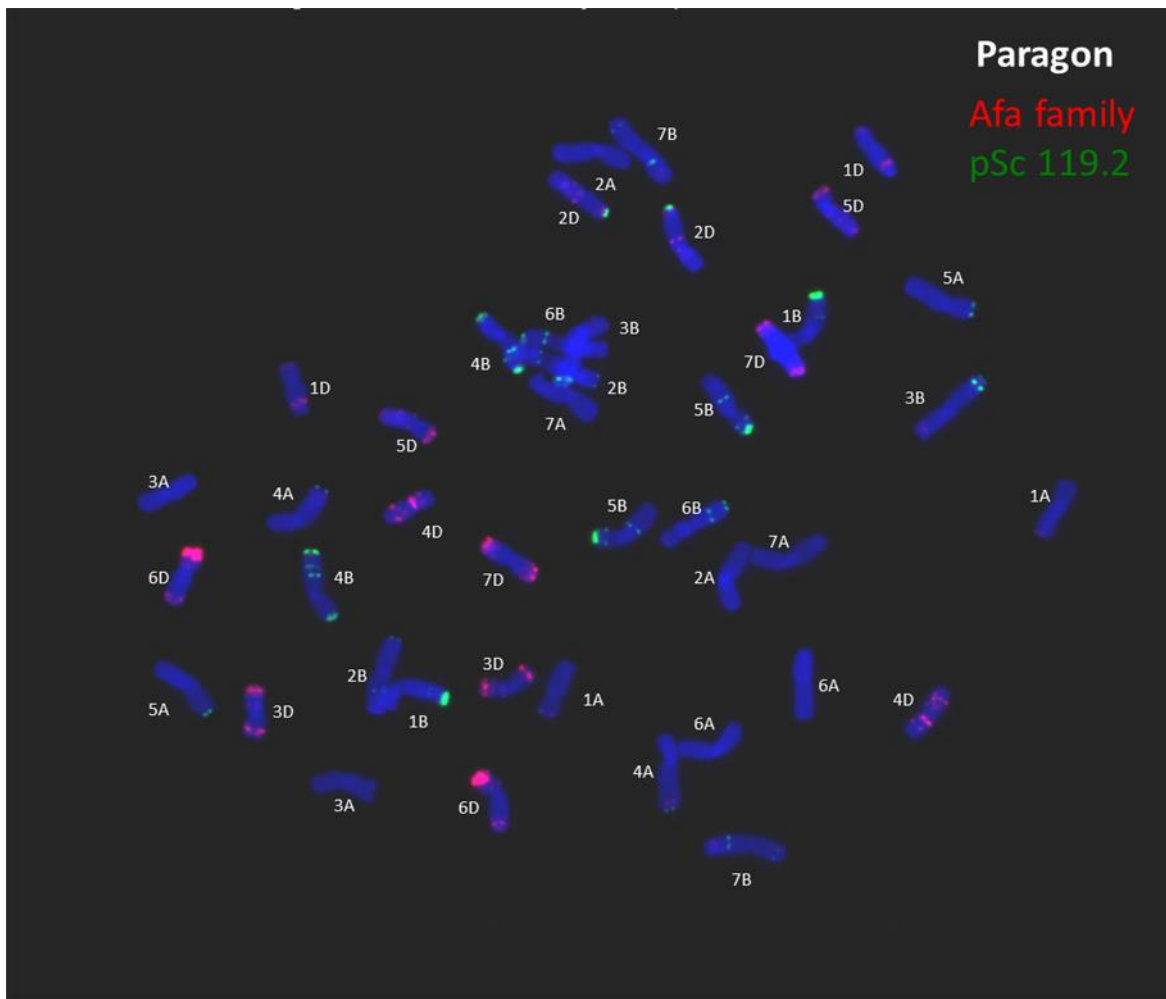


**Figure 4.4:** FISH with probe Afa-family (red signal) and pSc119.2 (green signal) on the metaphase spread of hexaploid wheat, cultivar Paragon.



### Introgression line genomes- Identification of introgressions using FISH

Identification of introgression using FISH was based on the green (pSc119.2) and red (Afa-family) signals present on the chromosomes. It was therefore difficult to identify small introgressions. If there were no signals present in the region of chromosome which contained the introgression it was impossible to identify the introgressions. Therefore, introgressions where the genetic map showed a complete chromosome introgressed from *T. timopheevii* into wheat were targeted for the FISH analysis. Hence, three BC<sub>2</sub> plants namely BC<sub>2</sub>-703, BC<sub>2</sub>-637A and BC<sub>2</sub>-578D and seven BC<sub>3</sub> plants (BC<sub>3</sub>-376A, BC<sub>3</sub>-273A, BC<sub>3</sub>-368A, BC<sub>3</sub>-369C, BC<sub>3</sub>-378B, BC<sub>3</sub>-100C and BC<sub>3</sub>-93B) were analyzed by fluorescent *in situ* hybridization (Figure 4.5, 4.6, 4.7, 4.8, 4.9, 4.10, 4.11, 4.12, 4.13 and 4.14). In each of the genotypes observed, the number of *T. timopheevii* introgressions identified by the SNP analysis corresponded exactly with the number of introgressions detected by FISH (Table 4.7). In the selected genotypes, all the chromosomes could be identified using Afa-family and pSc119.2 FISH probes. Chromosomes identification and counting was also carried out on metaphase spreads. Most of the backcross lines of *T. aestivum* x *T. timopheevii* examined were found to have different numbers of chromosomes. Out of the 10 backcross lines examined, two BC<sub>3</sub> lines (BC<sub>3</sub>-273A and BC<sub>3</sub>-93B) contained 42 chromosomes, five lines (BC<sub>3</sub>-376A, BC<sub>2</sub>-703A, BC<sub>2</sub>-637A, BC<sub>3</sub>-369C and BC<sub>3</sub>-378B) contained 41 chromosomes and three lines (BC<sub>2</sub>-578D, BC<sub>3</sub>-368A and BC<sub>3</sub>-100C) contained 40 chromosomes, (Table 4.7).



**Table 4.7:** Number of introgressed segments from *T. timopheevii* present in backcross plants as detected by SNP genotyping and fluorescence *in situ* hybridisation (FISH).

| Accession of backcross plants | Introgressions                                                          |                                                                                   | FISH                     |                                                                              |                                |                                                 |                                                                  | #chromosomes | Mapping and FISH |
|-------------------------------|-------------------------------------------------------------------------|-----------------------------------------------------------------------------------|--------------------------|------------------------------------------------------------------------------|--------------------------------|-------------------------------------------------|------------------------------------------------------------------|--------------|------------------|
|                               | Whole chromosome                                                        | Arm                                                                               | Addition                 | Substitution                                                                 | Introgression                  | Another copy missing                            | No or same signal                                                |              |                  |
| BC <sub>2</sub> -637A         | LG3G, LG4A <sup>t</sup><br>LG5G, LG6A <sup>t</sup><br>LG7A <sup>t</sup> | LG1G/1B<br>-short arm<br>LG3A <sup>t</sup> /3A<br>-long arm<br>LG2G-<br>short arm |                          | LG3G, LG6A <sup>t</sup> ,<br>LG4A <sup>t</sup> , LG7A <sup>t</sup> ,<br>LG5G | One copy of<br>1G/1B,<br>2G/2B | One copy of 6B, 6D missing                      | 3A <sup>t</sup> -same signal as 3A for introgression part        | 41           | Agree            |
| BC <sub>3</sub> -93B          | LG6G                                                                    | LG2A <sup>t</sup> -<br>short arm<br>LG3G-<br>short arm                            | 2 copies of 6G           |                                                                              | One copy of 3G/3B              | One copy of 6A missing                          | 2A <sup>t</sup> -no signal                                       | 42           | Agree            |
| BC <sub>3</sub> -378B         |                                                                         | LG2G-<br>short and long arm                                                       |                          |                                                                              | Three copies of 2G/2B          | One copy of 4D and 7D missing                   |                                                                  | 41           | Agree            |
| BC <sub>2</sub> -578D         | LG2G, LG4G                                                              | LG5A <sup>t</sup> -<br>short and long arm<br>LG6A <sup>t</sup> -<br>long arm      | LG2G                     | LG4G                                                                         |                                | One copy of 1A <sup>t</sup> , 5B and 7D missing | 6A <sup>t</sup> -no signal<br>5A <sup>t</sup> -same signal as 5A | 40           | Agree            |
| BC <sub>3</sub> -273A         |                                                                         | LG5A <sup>t</sup> -<br>long arm                                                   |                          |                                                                              |                                |                                                 | 5A <sup>t</sup> -same signal as 5A for introgression part        | 42           | Agree            |
| BC <sub>3</sub> -368A         | LG2G, LG7G                                                              | LG6A <sup>t</sup> -<br>long arm                                                   | 2 copies of LG2G<br>LG7G |                                                                              |                                |                                                 | 6A <sup>t</sup> -no signal for introgression part                | 40           | Agree            |
| BC <sub>3</sub> -369C         |                                                                         | LG2G not on small portion of                                                      | LG2G                     |                                                                              |                                | One copy of 2A, 6D missing                      |                                                                  | 41           | Agree            |

|                       |                   |                                                                                           |      |                   |                      |                                                            |                                                          |    |       |
|-----------------------|-------------------|-------------------------------------------------------------------------------------------|------|-------------------|----------------------|------------------------------------------------------------|----------------------------------------------------------|----|-------|
|                       |                   | short arm<br>LG7A <sup>t</sup> -<br>short arm                                             |      |                   |                      |                                                            |                                                          |    |       |
| BC <sub>3</sub> -376A | LG3A <sup>t</sup> | LG2G, LG7<br>A <sup>t</sup> -short<br>arm<br>LG5A <sup>t</sup> -<br>short and<br>long arm |      | LG3A <sup>t</sup> | One copy of<br>2G/2B | One copy<br>of<br>1A <sup>t</sup> , 1D, 3A<br>missing      | 5A <sup>t</sup> -no signal<br>7A <sup>t</sup> -no signal | 41 | Agree |
| BC <sub>3</sub> -100C | -                 | -                                                                                         | -    | -                 | -                    | One copy<br>of 5D, 7D<br>missing                           | -                                                        | 40 | Agree |
| BC <sub>2</sub> -703A | LG1A <sup>t</sup> | LG2G on<br>short and<br>long arm<br>LG7A <sup>t</sup> on<br>short arm                     | LG2G | LG1A <sup>t</sup> |                      | One copy<br>of 3D, 5D<br>and 7D<br>missing<br>One<br>copy? | 7A <sup>t</sup> -no signal                               | 41 | Agree |

#### **4.3.3 Comparison of *T. timopheevii* and *T. aestivum***

The *T. timopheevii* maps constructed in the following study were compared with the IWGSC positions of wheat and showed that the SNP markers from the A<sup>t</sup> genome of *T. timopheevii* mainly had synteny with the A genome of wheat and the SNP markers mapped to the G genome had synteny to the B genome of wheat. Individual instances of transfer of a group of markers (LG1G and LG6A<sup>t</sup>) or individual markers from one chromosome to another were recorded. The order of markers remained the same in the region of the non homoeologous translocation 5AL/4AL (5A<sup>t</sup>L/4A<sup>t</sup>), inherited by the polyploidy wheats belonging to the Emmer and Timopheevii groups from *T. urartu*.

**Figure 4.5:** SNP characterization of *T. timopheevii* introgressions in BC<sub>2</sub>-637A and FISH patterns on mitotic chromosomes of the BC<sub>2</sub>-637A using labeled repetitive total genomic probes, showing introgressions.

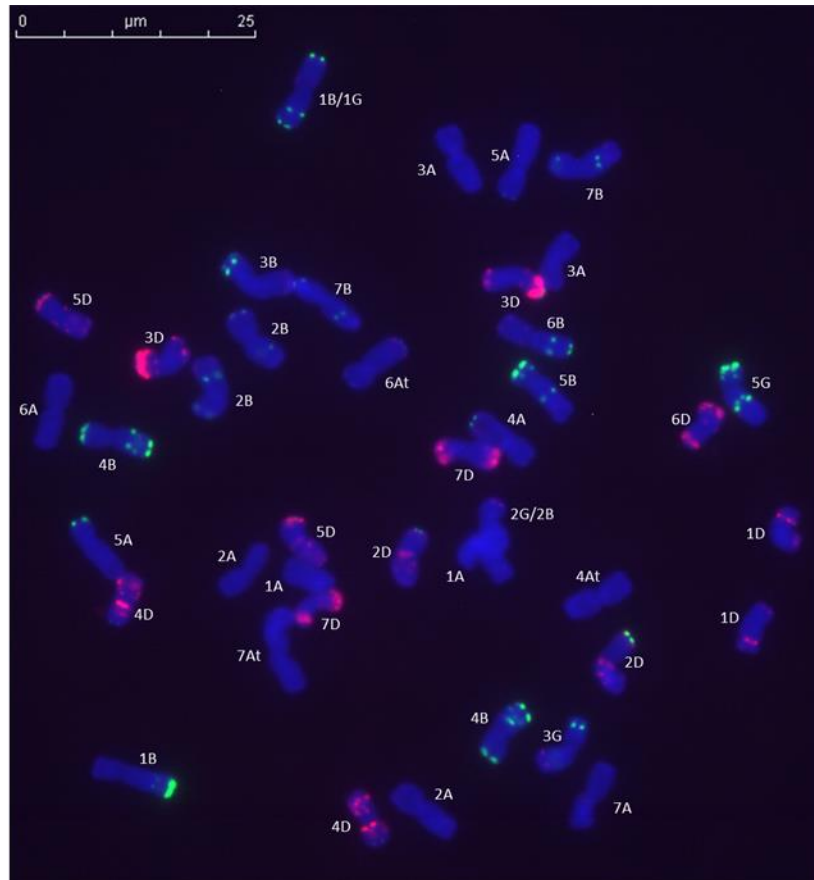
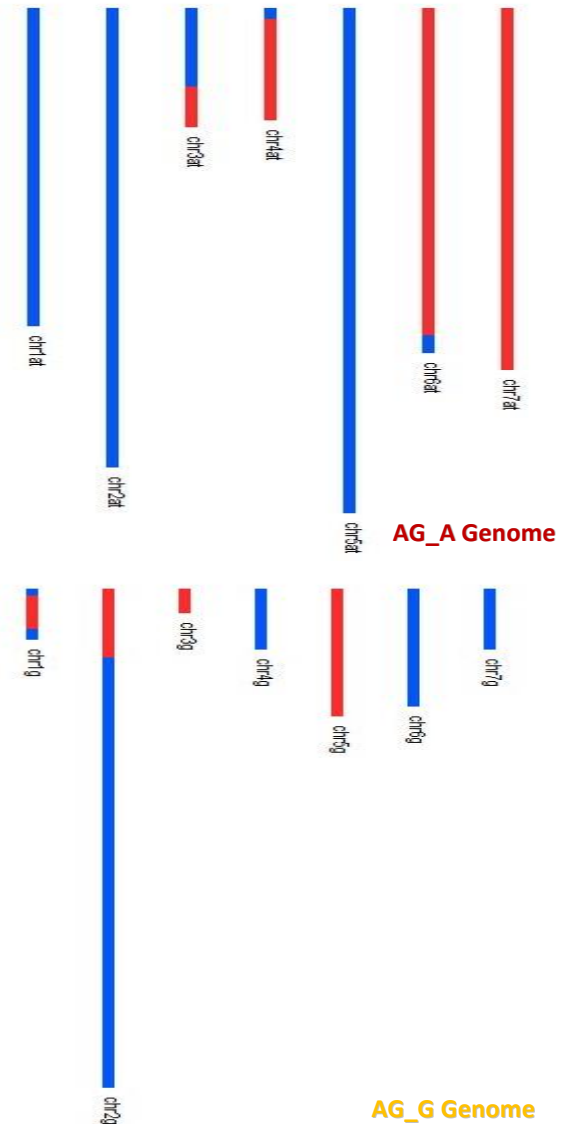


**Figure 4.6:** SNP characterization of *T. timopheevii* introgressions in BC<sub>3</sub>-376A and FISH patterns on mitotic chromosomes of the BC<sub>3</sub>-376A using labeled repetitive total genomic probes, showing introgressions.

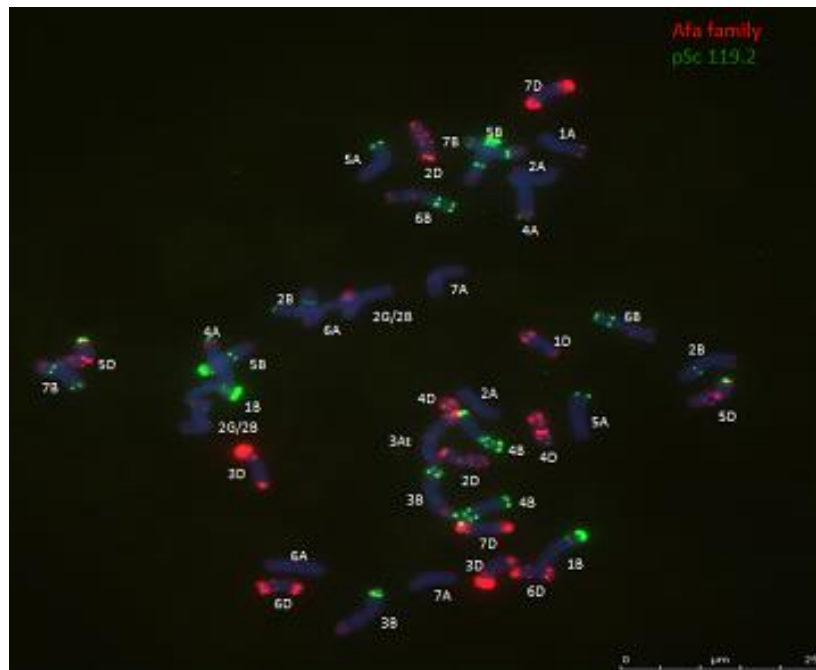
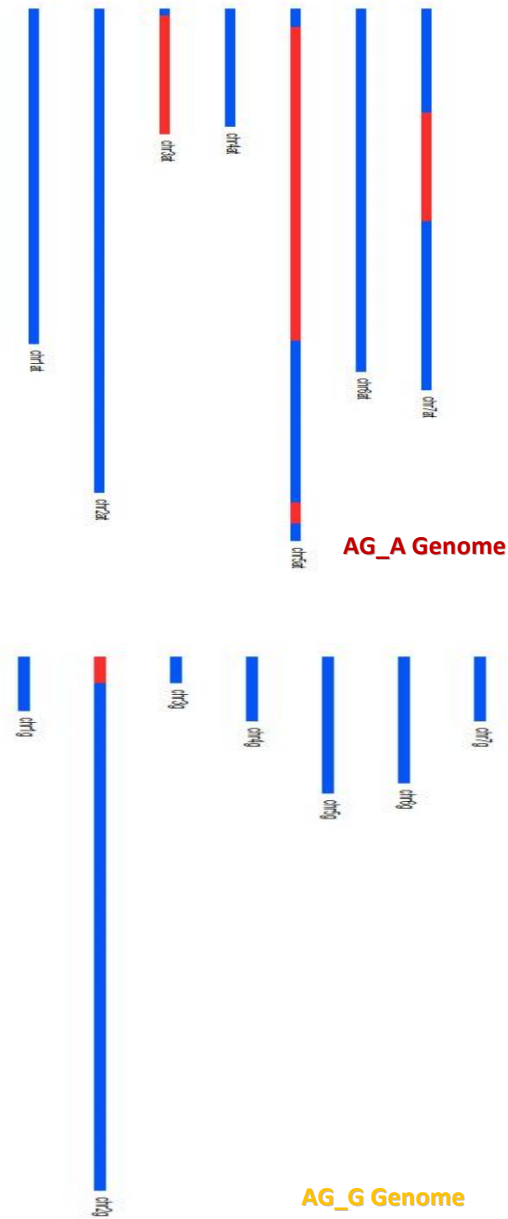


**Figure 4.7:** SNP characterization of *T. timopheevii* introgressions in BC<sub>3</sub>-93B and FISH patterns on mitotic chromosomes of the BC<sub>3</sub>-93B using labeled repetitive total genomic probes, showing introgressions.

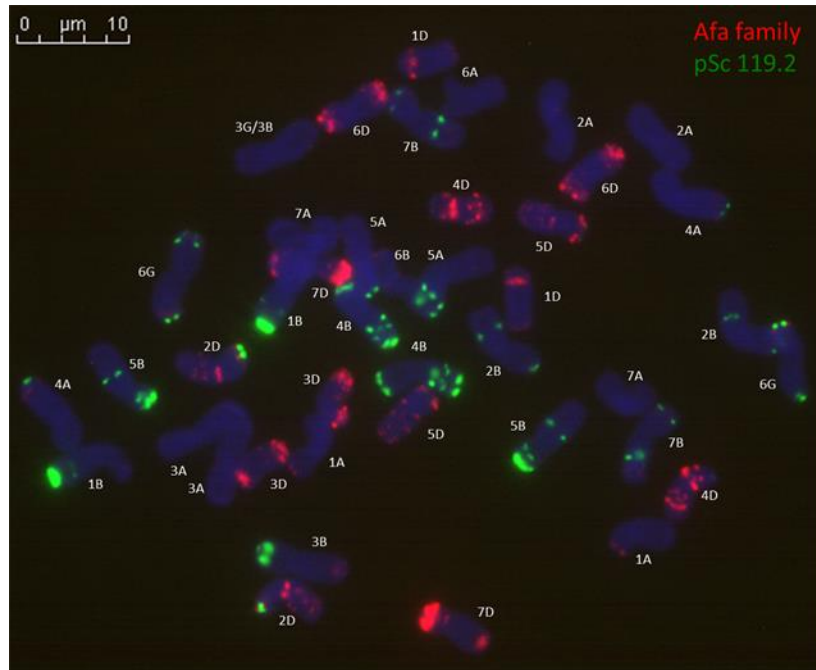
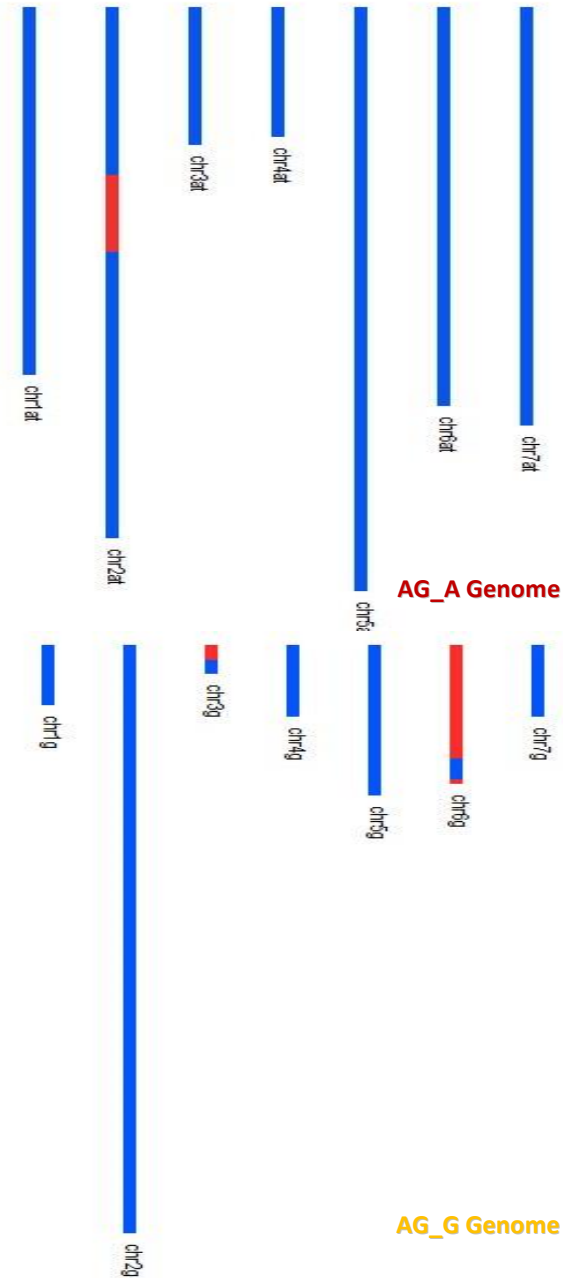


**Figure 4.8:** SNP characterization of *T. timopheevii* introgressions in BC<sub>3</sub>-378B and FISH patterns on mitotic chromosomes of the BC<sub>3</sub>-378B using labeled repetitive total genomic probes, showing introgressions.

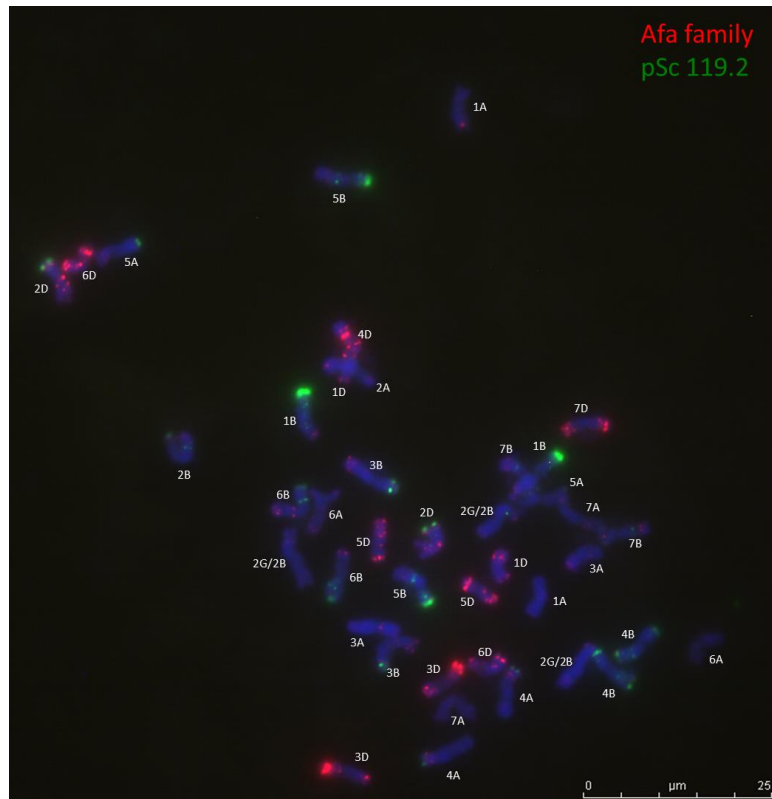
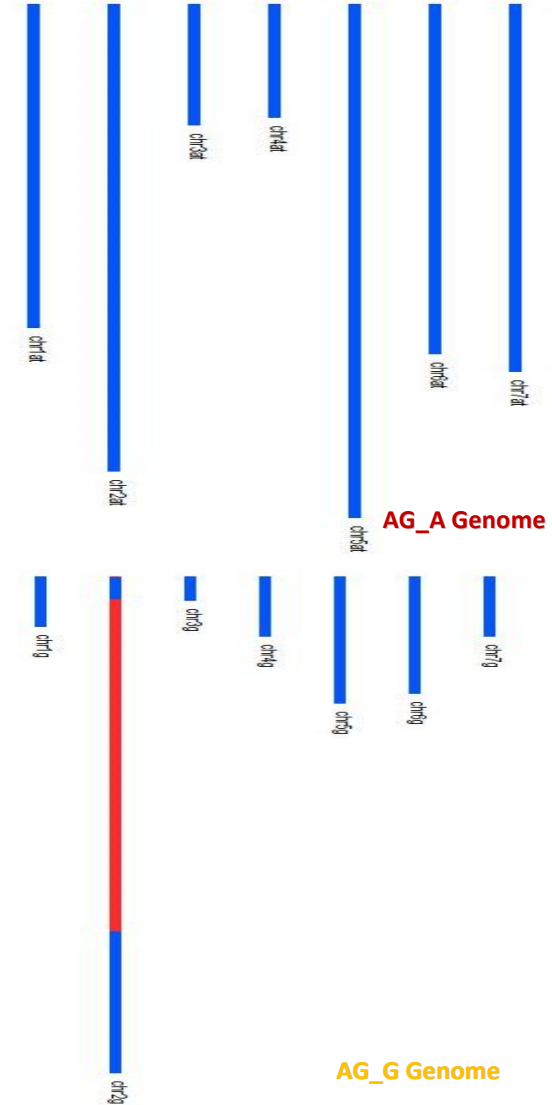


**Figure 4.9:** SNP characterization of *T. timopheevii* introgressions in BC<sub>2</sub>-578D and FISH patterns on mitotic chromosomes of the BC<sub>2</sub>-578D using labeled repetitive total genomic probes, showing introgressions.

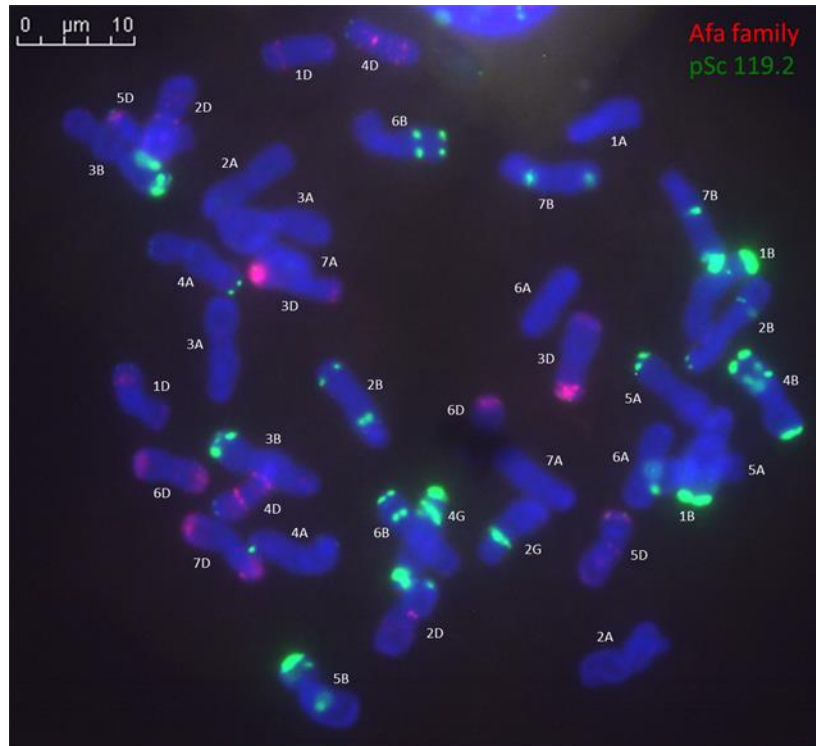
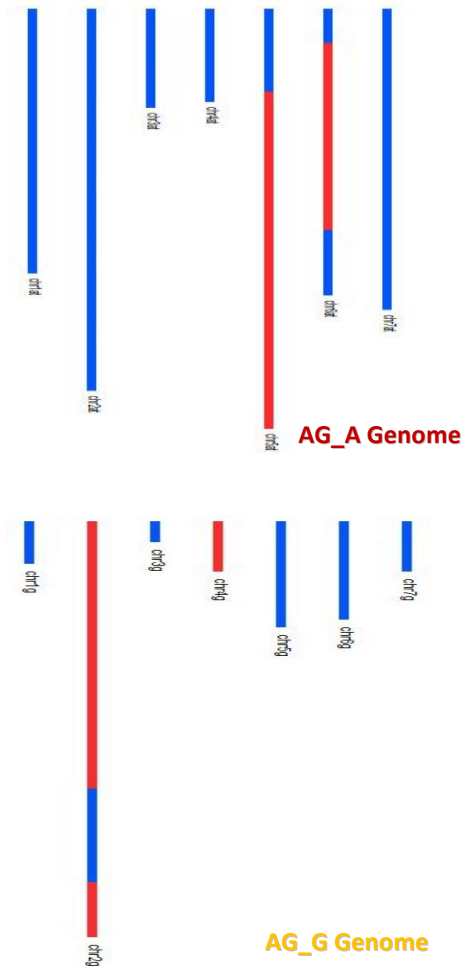




**Figure 4.10:** SNP characterization of *T. timopheevii* introgressions in BC<sub>3</sub>-273A and FISH patterns on mitotic chromosomes of the BC<sub>3</sub>-273A using labeled repetitive total genomic probes, showing introgressions.

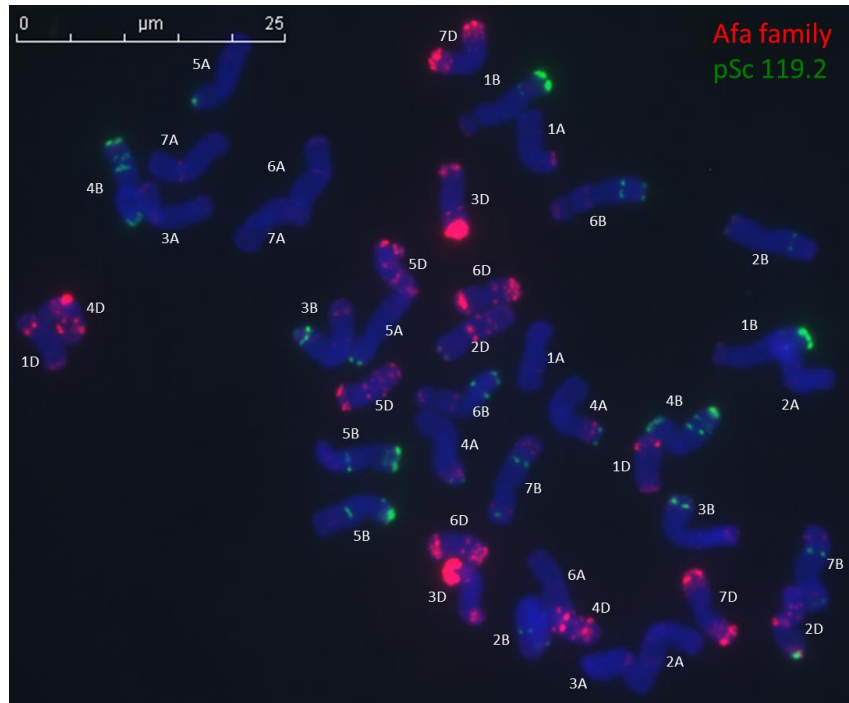
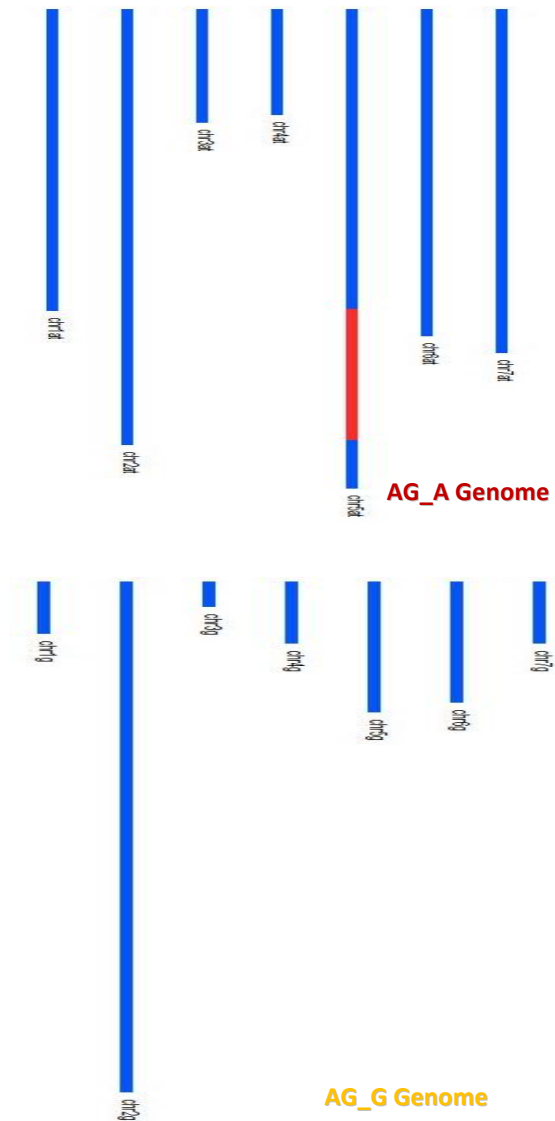


**Figure 4.11:** SNP characterization of *T. timopheevii* introgressions in BC<sub>3</sub>-368A and FISH patterns on mitotic chromosomes of the BC<sub>3</sub>-368A using labeled repetitive total genomic probes, showing introgressions.

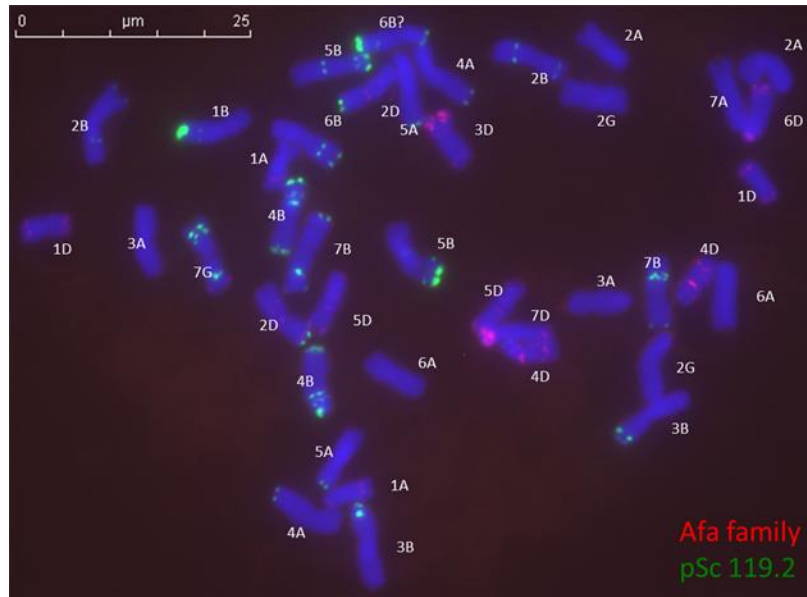
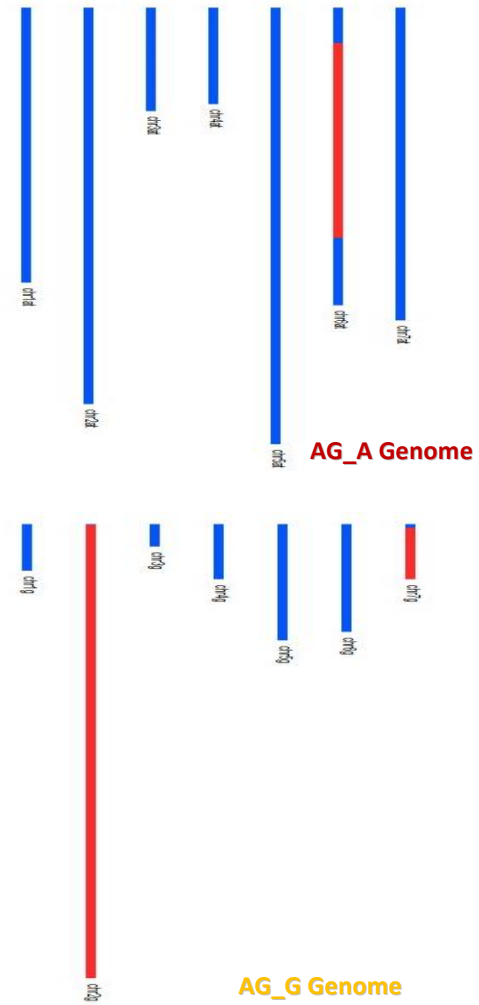


**Figure 4.12:** SNP characterization of *T. timopheevii* introgressions in BC<sub>3</sub>-369C and FISH patterns on mitotic chromosomes of the BC<sub>3</sub>-369C using labeled repetitive total genomic probes, showing introgressions.

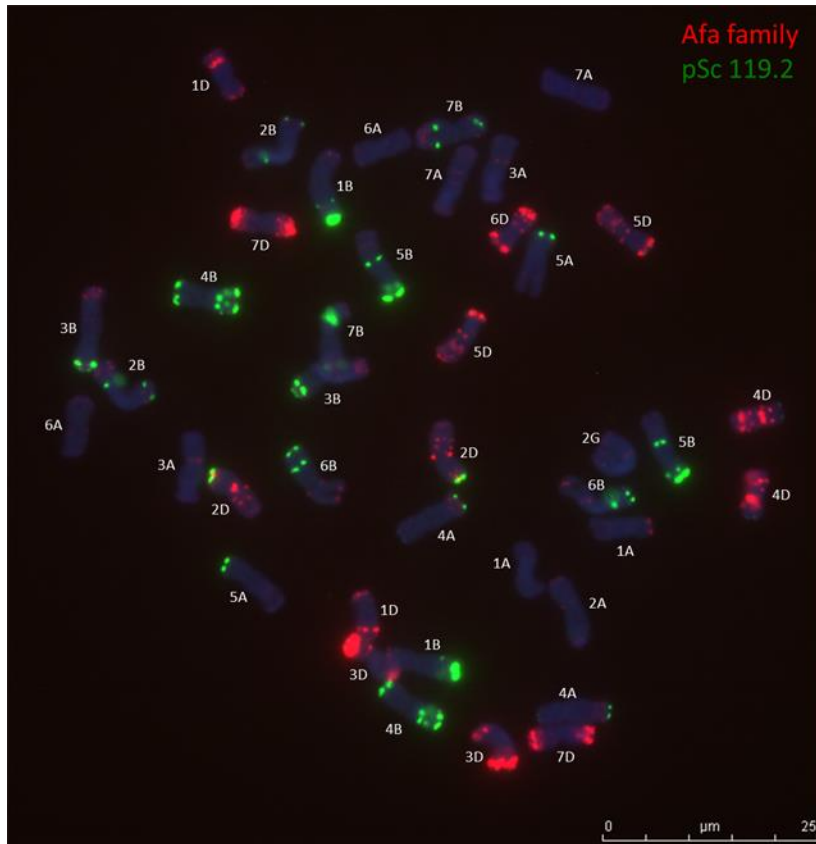
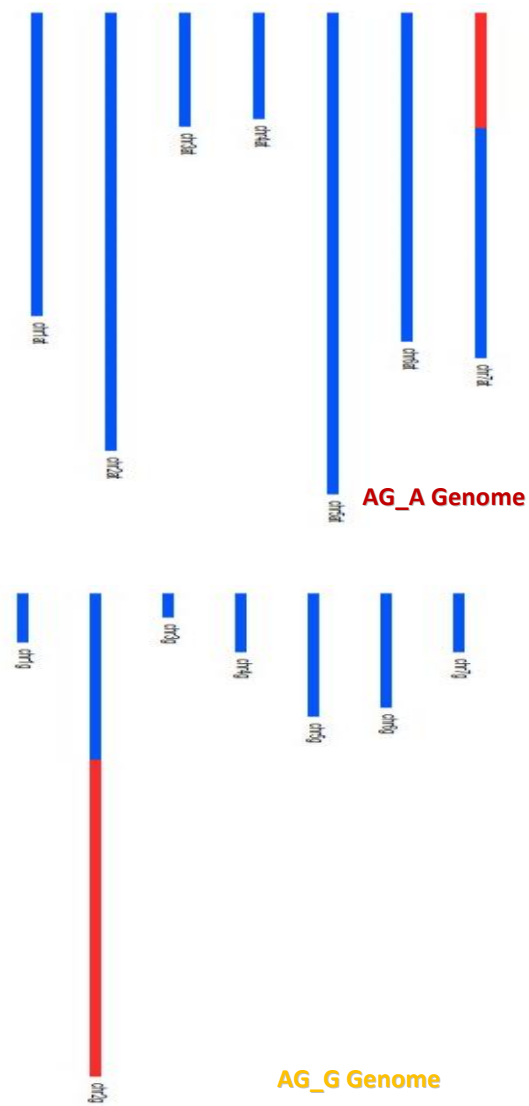


**Figure 4.13:** SNP characterization of *T. timopheevii* introgressions in BC<sub>3</sub>-100C and FISH patterns on mitotic chromosomes of the BC<sub>3</sub>-100C using labeled repetitive total genomic probes, showing introgressions.

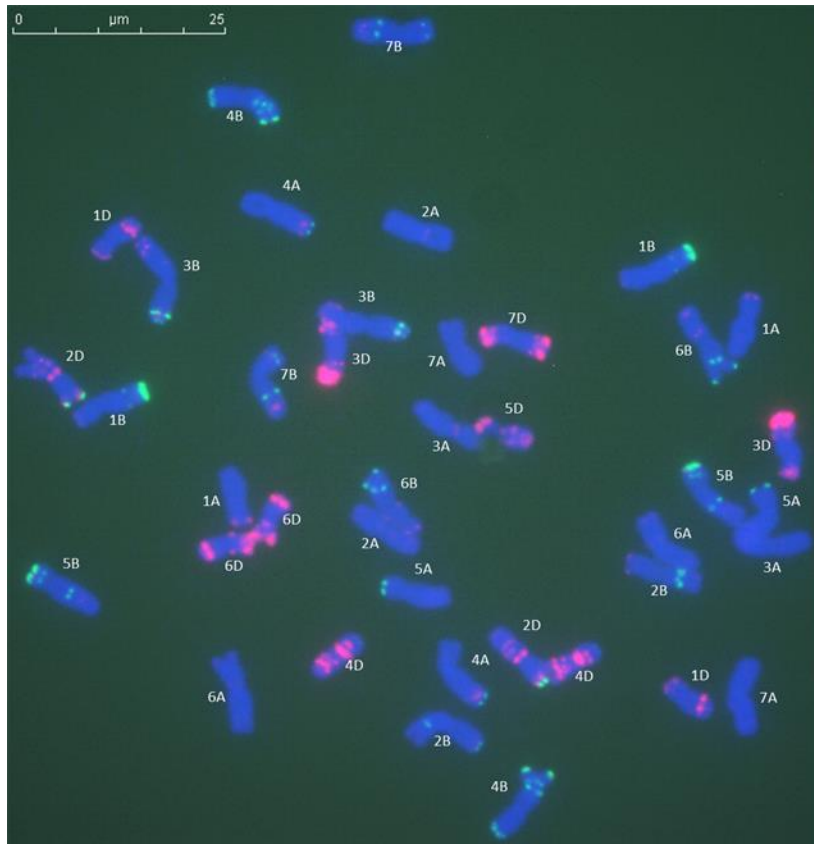
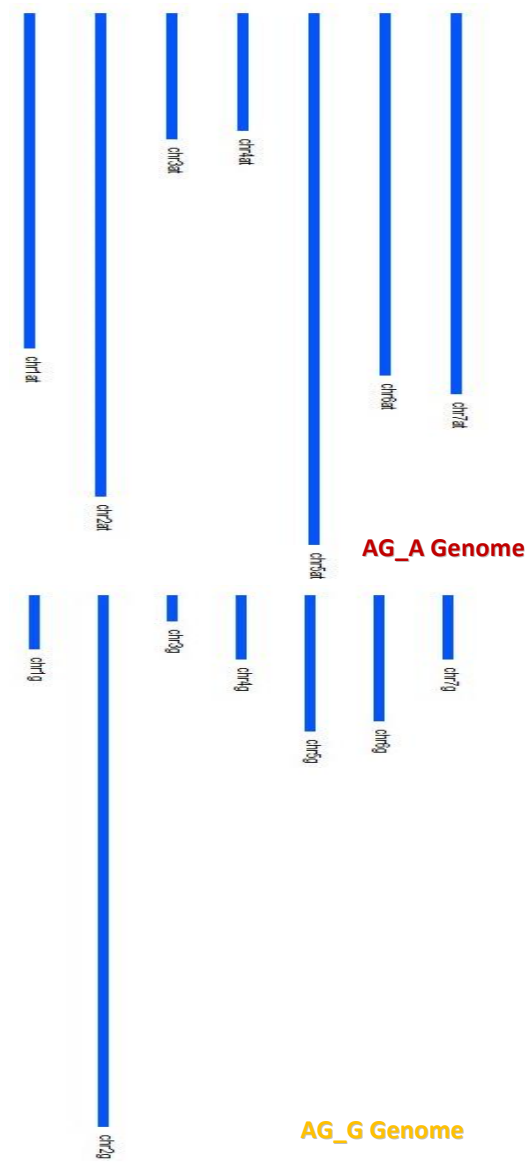
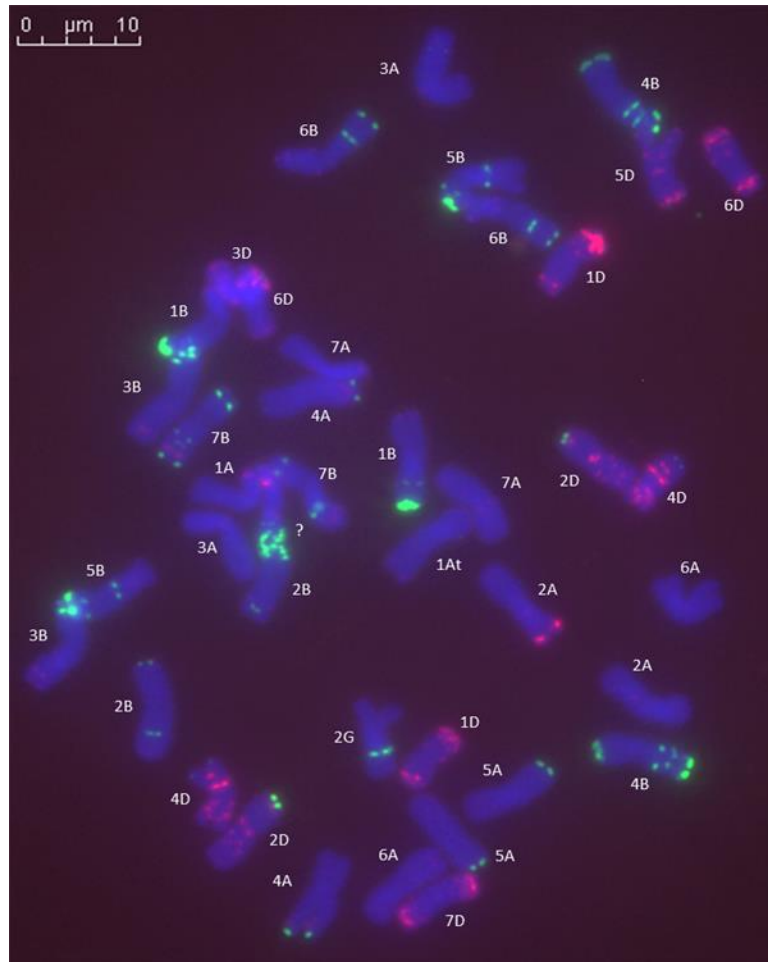
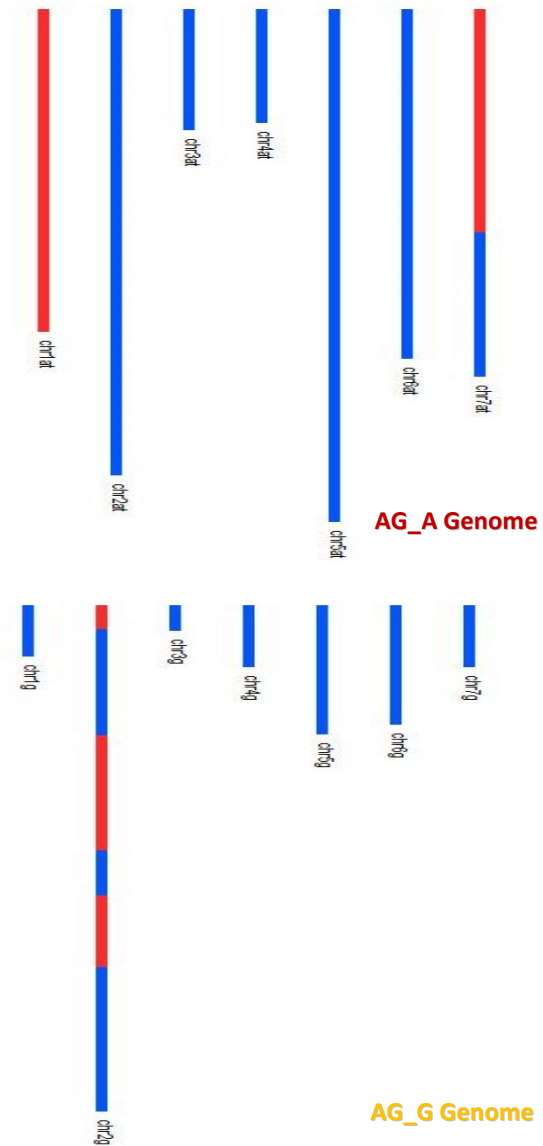


**Figure 4.14:** SNP characterization of *T. timopheevii* introgressions in BC<sub>2</sub>-703A and FISH patterns on mitotic chromosomes of the BC<sub>2</sub>-703A using labeled repetitive total genomic probes, showing introgressions.



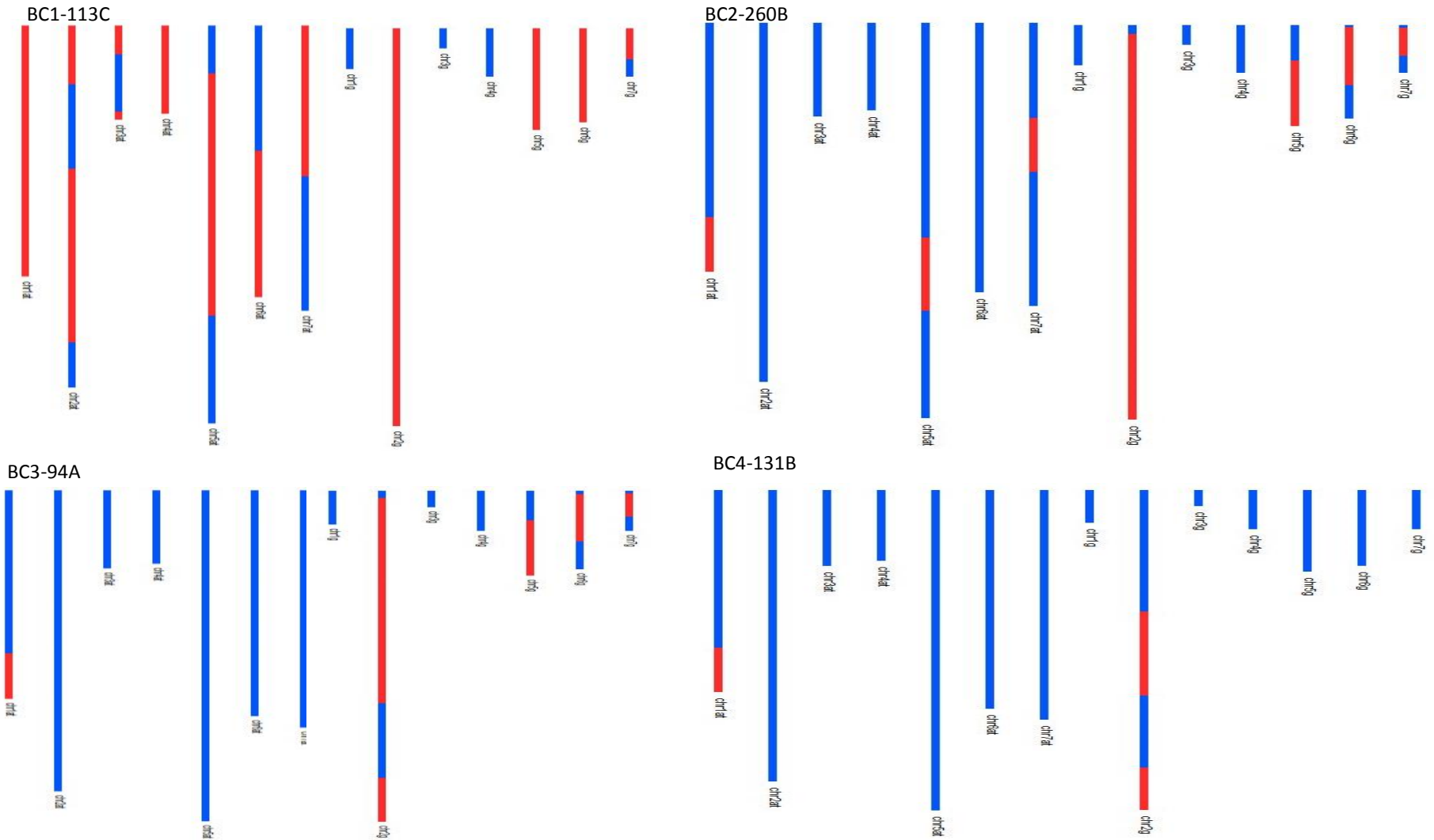
#### **4.4 Discussion**

The advent of next generation sequencing technologies as the Affymetrix wheat/wild relative array used in this present study enabled the identification and characterization of genome wide introgressions. Hence, this SNP array, having a large number of polymorphic markers has the ability to detect wheat/*T. timopheevii* recombinant chromosomes.

##### Genetic mapping

The total number of introgressions that could be generated is expected to be limited to those present in the 35 female F<sub>1</sub> gametes that gave rise to the 35 BC<sub>1</sub> plants. However, in contrast to the hypothesis, genetic mapping indicated that a very high frequency of interspecific recombination had occurred between the chromosomes of wheat and *T. timopheevii* in the later generations as well, that is in the gametes of the BC<sub>1</sub>, BC<sub>2</sub> and BC<sub>3</sub> generations (Figure 4.15). A total of 1,807 introgressions were generated covering the entire genome of *T. timopheevii*. The genetic map of A<sup>t</sup> and G genome of *T. timopheevii*, allowed the characterization and tracking of the introgressions (Figure 4.1 and Figure 4.2). However, the cM distances of the linkage groups should be treated with caution as the germplasm used to generate these linkage maps did not constitute proper mapping families and in fact different generations were combined in order to have sufficient numbers.

**Figure 4.15:** SNP analysis of *T. timopheevii* introgressions in four consecutive generations showing recombination had occurred during gametogenesis in the BC<sub>3</sub> genotype. Red colour shows the presence of introgressions, and blue colour shows wheat chromosomes.



The number of introgressions was mostly higher for the A<sup>t</sup> genome than the G genomes of *T. timopheevii* (Table 4.5). These results were supported by the findings of Feldman in 1966 where he analyzed F<sub>1</sub> hybrids from the cross between *T. timopheevii* and *T. aestivum* and found that chromosomes of the B and G genomes conjugated in 30% of cases, and the chromosomes of the A and A<sup>t</sup> genomes, in 70% of the cases. As discussed in the introduction, the A<sup>t</sup> genome of *T. timopheevii* and the A genome of wheat are less diverged than the B and G genomes. Thus the number of introgressions was expected to be higher for the A<sup>t</sup> genome rather than the G genome due to the higher level of recombination. The exception to the above was chromosome 2G which was present in the highest number. This can be explained by a gametocidal gene on the 2G chromosome which results in a preferential transmission of the 2G chromosome. Similar findings for the preferential transmission of 2G chromosome owing to the gametocidal effect were found in previous studies (Timonova *et al.*, 2012a; Nyquist, 1962). The intervarietal 'Timgalen' X 'RL4137' cross revealed that the *T. timopheevii* chromosome segment was introgressed into chromosome 2B in the hybrid 'Timgalen' at a high frequency (Devos *et al.*, 1993). Recombination was also shown to be greatly reduced in the segment. It may well be that a gametocidal gene is also present on chromosome 6G, as the number of introgression fragments retained decreased to only a small extent with each backcross, i.e. 42.53% introgressions in BC<sub>2</sub>, 30.34% in BC<sub>3</sub> and 27.27% in BC<sub>4</sub> in LG6G (Table 4.5). These findings were supported by Badaeva *et al.* (2000, 2010) where it was proposed that chromosome 6G exhibit high compensation ability and are preferentially transferred via gametes, as it was detected in 53% of hybrids between *T. aestivum* x *T. timopheevii* and was



also found to be present in several wheat cultivars from Europe and Australia (Hohmann *et al.*, 1996). Hence the 2G and 6G chromosomes of *T. timopheevii* appear to have the ability for preferential transmission through the gametes due to the presence of gametocidal genes on these chromosomes where the level of transmission of 2G chromosome was found to be higher than that of chromosome 6G (Table 4.5).

Gametocidal genes have been mapped on chromosomes of the second homoeologous group of several Triticeae species e.g. chromosome 2S of *Ae. speltoides* (Brown-Guedira *et al.*, 1996; McIntosh *et al.*, 1998; Tsujimoto *et al.*, 1995). Marais and Pretorius, (1996) found that the level of transmission of the gametocidal genes from *Ae. speltoides* ssp. *ligustica* which came with a stem rust resistance gene was 100%, thus despite their extensive attempts to disrupt the *Gc* system through irradiation and mutagen (N-nitroso-N-methyl-urea) treatment, hybrids lacking the *Gc* gene(s) could not be recovered. In comparison to the gametocidal genes of Triticeae species where the level of transmission is 100%, the strength of gametocidal gene(s) from 2G and 6G chromosomes of *T. timopheevii* is not 100% despite of high level of transmission as single introgressions from other chromosomes were also found in the present study.

The present SNP analysis does not reveal which wheat chromosomes have been involved in introgressions with *T. timopheevii*. Thus, it is presently not possible to determine whether the introgressions of the A<sup>t</sup> and G genomes of *T. timopheevii* happened with the A, B or D genomes of wheat. SNP markers from the 35K array are currently being converted into KASP markers. These markers will allow us to track individual introgressions through the process of backcrossing and selfing, i.e.

to tag the introgressions with selected markers. The design of KASP markers is such that they will be wheat chromosome specific which will hence allow us to detect which wheat chromosome the introgressions has recombined with, i.e. A, B or D genomes of wheat.

#### In situ hybridisation

*Triticum timopheevii* somatic chromosomes were earlier identified using the N-banding and C-banding (Hutchinson *et al.*, 1982; Badaeva *et al.*, 1994b) techniques. However detailed chromosome descriptions of *T. timopheevii* were also obtained by Jiang and Gill, (1994a), Rodriguez *et al.*, (2000a) and Uhrin *et al.*, (2012) giving the C-banding and N-banding patterns of chromosomes of *T. timopheevii*. Fluorescence *in situ* hybridization (FISH) using repetitive DNA clones is a powerful tool for identifying chromosomes within a species (Bedbrook *et al.*, 1980; Rayburn and Gill, 1986a, b; Mukai *et al.*, 1993; Pedersen and Langridge, 1997) or for tracking intergenomic chromosome rearrangements in a polyploidy species (Linc *et al.*, 1999). The identification of *T. timococcum* chromosomes using FISH was previously reported by Miko *et al.* (2015). On the basis of these findings, the karyotype was developed for the two sets of chromosomes originating from *T. timopheevii* (genome A<sup>t</sup> and genome G) using the repetitive DNA probes pSc119.2 and Afa-family. Similarly using work by Sepsi *et al.*, (2008), a metaphase spread of wheat (cv. Paragon) was labelled for the three sets of wheat chromosomes, i.e. A, B and D, using the same set of probes. The distribution of highly repeated DNA sequences (pSc119.2 and Afa-family) used in this study was similar to Miko *et al.* (2015), indicating high karyotype stability of *T. timopheevii*. This was supported by Badaeva *et al.*, 1994 and Zoshchuk *et al.*, 2007, where cytogenetic analysis of 27

accessions of cultivated species of *T. timopheevii* was done and it was found that *T. timopheevii* wheats were characterized by a higher karyotype stability with respect to chromosomal aberrations and variation of the C-banding and FISH (pSc119.2, Spelt-1, Spelt-2) patterns, as compared to the wild progenitors. Low variability of electrophoretic spectra of storage proteins was also exhibited in *T. timopheevii* (Yakobashvili, 1989), again an indication of the stability of the *T. timopheevii* genome. The stability of the *T. timopheevii* genome is thought to be due to the fact that all of the available accessions were collected in a restricted geographical region.

A whole chromosome introgression of linkage group 6G, along with introgressions from the short arm of LG2A<sup>t</sup> and LG3G were identified via SNP genetic mapping in BC<sub>3</sub>-93B. The FISH karyotype of BC<sub>3</sub>-93B showed that the pSc119.2 repeat blocks had the same strong signals in the terminal region of linkage group 6 as *T. timopheevii* in contrast to the two strong signals usually seen on the long arm of chromosome 6B of wheat. For the small introgression from 3G, the karyotype showed a chromosome with the same Afa-family signals on the short arm which covered one pSc119.2 signal along with two strong green signals of pSc119.2. This, confirmed the introgression of chromosome 3G. There was no signal present on chromosome 2A<sup>t</sup> for the introgressed part on the short arm so- it was impossible to detect this small introgression.

With the help of molecular genetics (SSR markers) and cytogenetic (C-banding and FISH) analysis, Timonova *et al.* (2012b) identified and characterized a complicated reorganization of the second homoeologous group of chromosomes during the creation of *T. aestivum*-*T. timopheevii* hybrid lines, as the hybrid line having a

2BS.2GL translocation displaced the 2D chromosome during backcrossing rather than the 2B chromosome as expected. This can also be seen in the present study in genotype BC<sub>3</sub>-369C, where there was a large single introgression from 2G as shown by SNP analysis. The 2G from *T. timopheevii* replaced either a 2A or a 6D chromosomes during backcrossing (not the 2B chromosome, as might be expected). This was evident from the presence of two copies of 2B along with one copy of 2G and the absence of one copy of each of 2A and 6D (Table 4.7). It would be expected that the 2G introgression would replace the 2A chromosome if the introgression was a compensating one. However, this could not be determined by the FISH signals. Greater coverage by FISH, e.g. by the use of more probes, may have been able to confirm which wheat chromosome was actually involved. Hence, confirming the complicated reorganization of 2G chromosome in *T. timopheevii*.

On the basis of study of 10 backcross introgression lines, it was observed that chromosomes 1A<sup>t</sup>, 2G, 3G, 5G had a high frequency of substitutions (Table 4.7). The chromosomes characterized with a high frequency of substitutions are supposed to have a high compensation capacity. Similar results were found by Liu *et al.*, 1992; Jiang and Gill, 1994a where they suggested that chromosomes 1A<sup>t</sup>, 2A<sup>t</sup>, 2G, 3G, 5G, 6G remain unmodified after species-specific translocations in the parental wheat species and therefore, have a high frequency of substitutions. Hence, it is reasonable to suppose that these *T. timopheevii* chromosomes are similar to their homoeologous chromosomes in wheat.

A complete set of 42 chromosomes was found in two backcross plants, BC<sub>3</sub>-273A and BC<sub>3</sub>-93B. Thus, these genotypes should be cytologically stable i.e. the meiosis will be normal and hence unstable gametes would not occur. However, the rest of

the backcross plants looked at with FISH, i.e. BC<sub>2</sub> (BC<sub>2</sub>-578D, BC<sub>2</sub>-368A, BC<sub>2</sub>-703A) and BC<sub>3</sub> (BC<sub>3</sub>-100C, BC<sub>3</sub>-637A, BC<sub>3</sub>-378B, BC<sub>3</sub>-369C, BC<sub>3</sub>-376A) had 40 or 41 chromosomes (Table 4.7), which will be cytologically unstable.

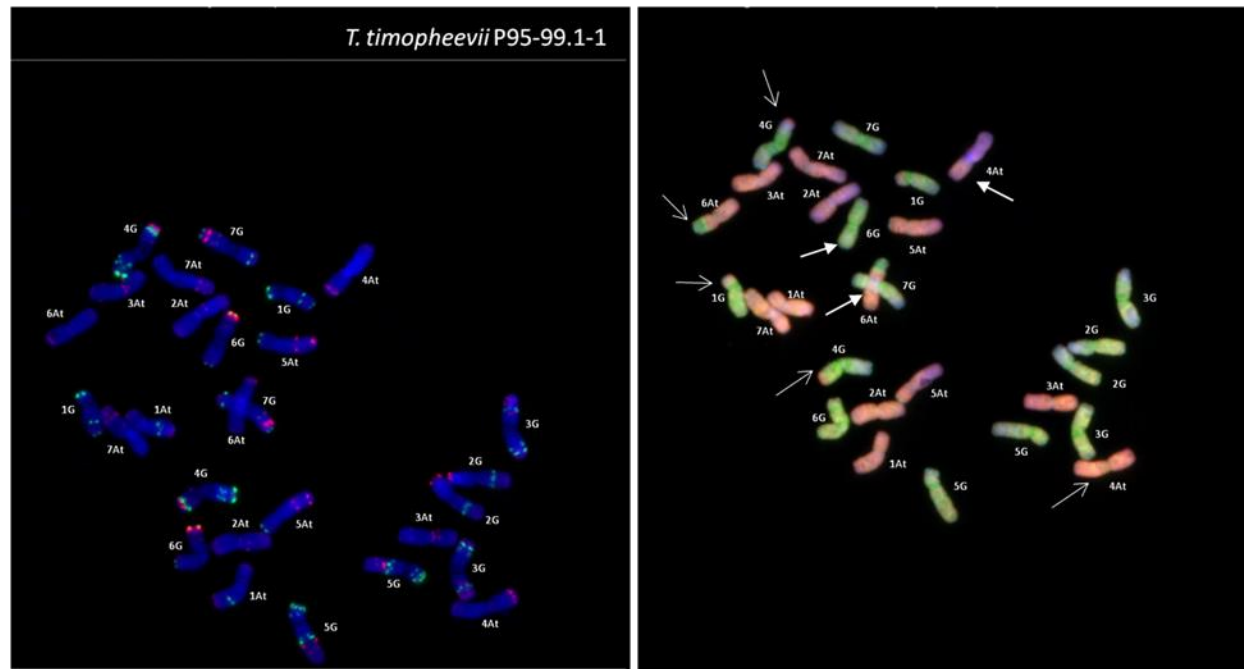
The present study proved that in a hybrid between *T. aestivum* and *T. timopheevii*, with a single whole chromosome of *T. timopheevii*, the *T. timopheevii* chromosome could be identified by fluorescence *in situ* hybridization (FISH) using a combination of DNA probes (Afa-family and pSc119.2). All seven A, B and D chromosomes of wheat could also be discriminated from each other with respect to the introgressions from *T. timopheevii*.

#### Chromosome structure of *T. timopheevii* relative to *T. aestivum*

The chromosome structure of *T. timopheevii* has been looked at by Badaeva *et al.*, (1994); Jiang and Gill, (1994a); Maestra and Naranjo, (1999) and Rodriguez *et al.*, (2000b) using cytogenetic methods including C-banding, sequential N-banding and GISH- These papers confirmed the presence of several translocations in the *T. timopheevii* accessions looked at including 6A<sup>t</sup>S/1GS, 1GS/4GS, 3A<sup>t</sup>L/4A<sup>t</sup>L and 4GS/4A<sup>t</sup>L. Translocation 6A<sup>t</sup>S/1GS was also confirmed previously by the biochemical studies performed by Brown-Guedira *et al.* in 1996. In his study, Gliadin electrophoresis determined that no α (alpha) gliadins corresponding to the gliadin proteins encoded by a gene on wheat chromosome 6A were present in the 6A<sup>t</sup> (6A) substitution line. Because the short arm of chromosome 6A<sup>t</sup> known to be involved in a cyclic translocation with chromosome 1G and 4G, the gliadin locus (*Gli-A2*) present on the original chromosome 6A<sup>t</sup> may have been transferred to either chromosome 1G or 4G. The location of gene orthologous to *Gli-A2* confirms that a portion of the short arm of 6A<sup>t</sup> was replaced by part of the short arm of

chromosome 1G. The *T. timopheevii* accession (P95-99.1-1) used in the present study carries the same species-specific translocations previously reported. The short arm of chromosome 6A<sup>t</sup> has a long translocated segment from the short arm of chromosome 1G, while 1G has a short segment from 6A<sup>t</sup> (6A<sup>t</sup>S/1GS) revealed by mcGISH (Figure 4.16). McGISH also revealed that 4GS has a tiny segment from the A<sup>t</sup> genome, which probably originated from the long arm of chromosome 4A<sup>t</sup> (4GS/4A<sup>t</sup>L), as demonstrated by earlier studies.

**Figure 4.16:** The chromosomes of *T. timopheevii* (accession P95-99.1-1) involved in intergenomic translocations (6A<sup>t</sup>S/1GS, 4GS/4A<sup>t</sup>L) identified by FISH and GISH, where the chromosomes involved are represented by arrows.



Further, the comparison of the *T. timopheevii* maps from the present study to the marker positions on wheat also revealed the presence of translocations. Our data confirmed the presence of the translocation 6A<sup>t</sup>S/1GS, where markers from the short arm of chromosome 1G mapped on the short arm of chromosome 6A<sup>t</sup>, while 1G has markers mapped on the short arm from chromosome 6A<sup>t</sup>.

The genetic mapping and comparative analysis also confirmed the 1GS/4GS and 3A<sup>t</sup>L/4A<sup>t</sup>L previously reported. An introgression of markers from chromosome 3A<sup>t</sup>L was found into the long arm of chromosome 4A<sup>t</sup> and the presence of markers from 1GS on chromosome 4GS.

*T. timopheevii* shares the 4AL/5AL translocation seen in wheat which is inherited from *T. urartu* (King *et al.*, 1994) but carries no structural change comparable with translocation 4AL/7BS (Jiang and Gill, 1994a; Maestro and Naranjo, 1999). The conserved order of markers on chromosome 5A and 5A<sup>t</sup> and 4A and 4A<sup>t</sup> of *T. aestivum* and *T. timopheevii* confirms the similarity of these chromosomes and the presence of 4A<sup>t</sup>L/5A<sup>t</sup>L translocation analogous to 4AL/5AL. Thus, SNP analysis revealed a close syntenic relationship between genomes of wheat and the A<sup>t</sup> and G genomes of *T. timopheevii*.



## **Chapter 5**

### **Mineral analysis in *T. timopheevii***

#### **5.1 Introduction**

There are 13 vitamins and 17 mineral nutrients which are essential for growth and metabolism to develop a healthy life (Grusak, 1999), particularly large amounts of carbohydrates, proteins, and lipids; small amounts of macroelements; and trace amounts of microelements such as Iron (Fe), Zinc (Zn), Copper (Cu), Iodine (I) and Selenium (Se) (Welch and Graham, 2004). Among all micronutrients which are essential for sustaining human health, Fe, Zn, I and vitamin A have been reported to be most at risk of malnutrition. While global cereal grain yields have increased dramatically since the green revolution (Borlaug, 1983), global food systems are not providing sufficient micronutrients, resulting in an increased prevalence of micronutrient deficiencies (Welch, 2002).

##### **5.1.1 Micronutrient malnutrition**

Micronutrient malnutrition i.e. the deficiency of micronutrients affects more than 40% of world population, especially in many developing nations. Worldwide, more than 3 billion people who live on staple crops suffer from it and is commonly known as "hidden hunger" (White and Broadley, 2009). 60-80% Iron (Fe), >30% Zinc (Zn), 30% iodine (I), 15% selenium (Se), and vitamin A deficiency was observed in about one-third of humans in all age groups especially women and children (Ghandilyan *et al.*, 2006), which directly increase the social healthcare costs, reduce economic productivity and increased rate of mortality and morbidity (Holtz and Brown, 2004; Kennedy *et al.*, 2003; Combs *et al.*, 2001; Cakmak, 2008). The reason of human mineral malnutrition is the relatively low content of cation minerals in plant based foods in combination with

the abundance of anti-nutrient compounds such as phytic acid (myo-inositol-1, 2, 3, 4, 5, 6-hexakisphosphate), fibre (e.g. cellulose), polyphenols (e.g. tannins), haemagglutinins (e.g. lectins) and micronutrient-like competitive heavy metals (e.g. Cd) (Graham *et al.*, 2001; Hurrell, 2004; Welch and Graham, 2004 and Guttieri *et al.*, 2006) that severely reduce their bioavailability. Further crops grown on soil lacking plant-available mineral elements is another reason for the edible portions having low mineral concentrations. Fertilisation of these crops increase leaf mineral concentrations and improves the yield but does not always increase mineral concentrations in seed or grain to the desired level (Frossard *et al.*, 2000; Graham *et al.*, 2001).

### **5.1.2 Solution to micronutrient malnutrition**

Micronutrient supplementation, food fortification and dietary diversification are the traditional efforts that have been focused on to solve this problem of micronutrient deficiency (White and Broadley, 2005). However, in the developing countries where people cannot afford meat and fish products with high micronutrient content, these methods are not sustainable. So, these people depend largely on cereals as their staple food, which provide only small amount of micronutrients and do not meet human nutrition needs (Shi *et al.*, 2008). Cereal crops could provide a significant increase in the overall micronutrient availability in human populations whose diets are dominated by staple crop consumption. Bouis. (2003) estimated that doubling of Fe density in a staple crop would result in a 50% increase in total Fe intake for a population relying heavily on a single food.

### **5.1.3 Biofortification**

In recent years, biofortification has been proposed as a solution to mineral malnutrition (Singh *et al.*, 2005). Biofortification is a process for increasing the bioavailable concentrations of essential elements in the edible portion of crops through agronomic interventions or genetic selection (White and Broadley, 2005). Biofortification of staple food along with the dietary diversification is the most cost effective and sustainable approach for the alleviation of micronutrient malnutrition (Stein *et al.*, 2007; Bouis and Welch, 2010) where the criteria for successful biofortified crop being high yielding and profitable, effective in improving the micronutrient status of humans and acceptable to farmers and consumers.

### **5.1.4 Approaches for biofortification**

There are two types of approaches followed for biofortification, genetic and agronomic. Genetic biofortification involves classical breeding approaches to characterise and exploit genetic variation for mineral content, as well as new approaches involving gene discovery and marker assisted breeding (Grusak, 2002). Thus it is a strategy that uses plant breeding techniques to produce staple food crops with higher micronutrient levels, reducing levels of anti-nutrients and increasing the levels of substances that promote nutrient absorption. Thus, it offers a sustainable solution to malnutrition problems by exploring natural genetic variation in wild relatives, synthetics and landrace for micronutrient uptake from the soil and translocation in to grain that can supplement micronutrient-deficient rural inhabitants with limited access to formal markets or health care systems (Velu *et al.*, 2014). Agronomic biofortification involves the application of fertilizer directly to the soil or to the

crops for various micronutrients. It is short term approach and offers a rapid solution to the problem of malnutrition and represents useful complementary approach to on-going breeding programs.

Although agronomic approach using micronutrient fertilizers is one of the common agricultural approach but it involves some technology and costs. Whereas, crop biofortification via traditional cross breeding offers a sustainable and low-cost way to provide essential micronutrients to people in both developing and developed countries. Biofortified staple foods may not deliver equally high levels of minerals and vitamins per day, compared to supplements or fortified food products, but they can increase micronutrient intake for the resource-poor people who consume them daily, and therefore complement existing approaches (Bouis *et al.*, 2011).

#### **5.1.5 Wheat- Major cereal crop**

Wheat (*Triticum aestivum* L., 2n=6x-42, AABBDD) is the world's most important crop with a world production of about 730 million tons harvested from an area of over 220 million ha (FAOSTAT, 2016). Wheat constitutes approximately 30% of the total cereals consumed in the world and hence is a major source of minerals (FAO, 2003). Wheat is the most important source of carbohydrate in a majority of countries. Wheat starch is easily digested, as is most wheat protein and contains minerals, vitamins and fats (lipids).

Wheat grain apart from micronutrients is also a good source of other beneficial nutrients which could be targeted by breeding programs to improve the nutritional quality of wheat based products. Grain bran is particularly rich in dietary fibre and vitamins (folic acid), which have been associated with a protective role for many chronic diseases including cardiovascular diseases and

type 2 diabetes (Jacobs *et al.*, 1999; Liu *et al.*, 1999). The bioactive compounds such as tocopherols, sterols and arabinoxylan fibre (Shewry *et al.*, 2010) are also present in wheat which improves the nutritional quality of wheat.

#### **5.1.6 Need to improve micronutrient concentrations in wheat**

Cultivated wheat contains very low levels of essential micronutrients and shows a narrow genetic variation. Therefore, it is imperative that wheat cultivars with improved micronutrient concentrations should be developed to alleviate malnutrition among people who consume mainly cereals. For the identification of useful variability for wheat biofortification major emphasis has been on the screening of wild progenitor species of wheat, as wild and primitive wheats represent a better and more promising genetic resource for high micronutrient levels compared to cultivated wheats (*T. aestivum* and *T. turgidum*) (White and Broadley, 2005). During past decades, CGIAR (HarvestPlus and CIMMYT) have initiated research to determine available genetic variability for grain mineral elements from wide range of germplasm in the genus *Triticum* and to study the feasibility of using them in future wheat breeding programs (HarvestPlus Brief, 2006). Few examples of the screening of wild germplasm and transfer of genetic variability from wild relatives for micronutrient into wheat cultivars in past are given in Table 5.1.

**Table 5.1:** Screening of range of wild relatives germplasm for genetic variability for micronutrient content and their successful transfer to wheat cultivars.

| <b>Germplasm</b>                                                                                                  | <b>Micronutrients</b>                   | <b>Other characteristics</b>                                                                                                                             | <b>Reference</b>                                                                        |
|-------------------------------------------------------------------------------------------------------------------|-----------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------|
| Landraces,<br><i>T. dicoccoides</i> ,<br><i>Ae. tauschii</i> ,<br><i>T. monococcum</i> and<br><i>T. boeoticum</i> | High Fe and Zn content in grains        | Micronutrient content of wild relatives higher than landraces                                                                                            | Ortiz-Monasterio <i>et al.</i> , 2007                                                   |
| Wild emmer wheat accessions                                                                                       | High Fe, Zn and protein in grains       | High tolerance to drought stress and Zn deficiency in soil                                                                                               | Peleg <i>et al.</i> , 2008                                                              |
| <i>Ae. longissima</i>                                                                                             | High grain micronutrient concentrations | Transferred to elite wheat cultivars (as SI genome being similar to B genome of hexaploid wheat)                                                         | Dvorak and Zhang, 1990; Friebe <i>et al.</i> , 1996                                     |
| <i>Ae. cylindrical</i><br><i>Ae. ventricosa</i><br><i>Ae. geniculata</i>                                          | High Fe and Zn content in grains        |                                                                                                                                                          | Friebe <i>et al.</i> , 1996                                                             |
| Diverse collection of <i>T. turgidum</i> ssp. <i>dicoccoides</i> substitution lines in various wheat backgrounds  | High Fe and Zn content in grains        | Associated with 6A, 6B and 5B substitution lines                                                                                                         | Cakmak <i>et al.</i> , 2004                                                             |
| <i>Ae. kotschy</i> backcross derivatives                                                                          | High Fe and Zn content in grains        | Associated with 2S and 7U chromosomes                                                                                                                    | Tiwari <i>et al.</i> , 2010                                                             |
| Wheat- <i>Ae. kotschy</i> substitution line with chromosome 7U <sup>k</sup>                                       | High Fe and Zn content in grains        | Genes present on 7U <sup>k</sup> chromosome are highly effective for uptake, translocation and mobilization of Fe and Zn contents in endosperm of grains | Kumar <i>et al.</i> , 2016                                                              |
| <i>T. dicoccoides</i> (recombinant substitution lines)                                                            | High protein and Zn concentrations      | Locus <i>Gpc-B1</i> on chromosome 6BS                                                                                                                    | Fahima <i>et al.</i> , 2006; Uuay <i>et al.</i> , 2006; Distelfeld <i>et al.</i> , 2007 |
| Wheat-rye                                                                                                         | High Zn content in addition             | 1R, 2R and 7R chromosomes of rye carry                                                                                                                   | Cakmak <i>et al.</i> , 1997                                                             |

|                                                                                        |                                     |                                                                                                                                                                |                                                               |
|----------------------------------------------------------------------------------------|-------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------|
| chromosome addition lines and their rye parents                                        | lines than their parents            | genes controlling Zn efficiency                                                                                                                                |                                                               |
| <i>T. monococcum</i> , <i>T. boeoticum</i> , <i>T. dicoccoides</i> and <i>T. durum</i> | High Zn content in grain            | 21 QTL reported on ten chromosomes for increased grain Zn concentration                                                                                        | Xu <i>et al.</i> , 2011; Velu <i>et al.</i> , 2014            |
| RIL population ( <i>T. monococcum</i> x <i>T. boeoticum</i> )                          | High Fe and Zn contents in grain    | QTL reported on chromosome 2A and 7A                                                                                                                           | Tiwari <i>et al.</i> , 2009                                   |
| RIL population ( <i>T. durum</i> x <i>T. dicoccoides</i> )                             | High Fe and Zn content in grain     | Three major QTL for grain Fe and Zn, two on chromosome 2A and one on 7A                                                                                        | Peleg <i>et al.</i> , 2009                                    |
| Rice                                                                                   | High Fe concentration               | Major QTL found on chromosome 8 which is syntenic to chromosome 7 of wheat                                                                                     | Stangoulis <i>et al.</i> , 2007; Singh <i>et al.</i> , 2004   |
| <i>Ae. kotschy</i> , <i>Ae. longissima</i> , <i>Ae. peregrine</i>                      | High Fe and Zn concentrations       | Homoeologous group 1, 2, 4 and 7 of <i>Ae. longissima</i> and group 7 of <i>Ae. peregrine</i>                                                                  | Rawat <i>et al.</i> , 2011; Neelam <i>et al.</i> , 2011, 2013 |
| Bread wheat<br>Durum wheat<br><i>T. monococcum</i>                                     | High Fe and Zn concentrations       | Group 2 and 7 chromosomes and in some cases on group 4 and 5 chromosomes                                                                                       | Ozkan <i>et al.</i> , 2007; Genc <i>et al.</i> , 2008         |
| <i>Ae. biuncialis</i>                                                                  | High K, Zn and Mn content in grains | Chromosome 3M <sup>b</sup>                                                                                                                                     | Farkas <i>et al.</i> , 2014                                   |
| RIL population (PBW 343 x Kenya Swara)                                                 | High Zn content                     | Novel QTL found on chromosome 2B and 3A, introgressed into elite background                                                                                    | Hao <i>et al.</i> , 2014                                      |
| <i>Ae. tauschii</i> (synthetic hexaploid)                                              | High Zn content in grains           | Released biofortified wheat 'Zinc Shakti' (Chitra) developed by introgressing synthetic hexaploid with elite germplasm which accounted for 40% higher grain Zn | Velu <i>et al.</i> , 2015                                     |

|                                                                                       |                        |                                                                                     |                         |
|---------------------------------------------------------------------------------------|------------------------|-------------------------------------------------------------------------------------|-------------------------|
| 12 accessions of <i>T. timopheevii</i> , Chinese spring, Durum wheat cultivar Langdon | High Fe and Zn content | High micronutrient content in <i>T. timopheevii</i> than Chinese spring and Langdon | Hu <i>et al.</i> , 2017 |
|---------------------------------------------------------------------------------------|------------------------|-------------------------------------------------------------------------------------|-------------------------|



The useful variability for wheat biofortification among various wild relatives listed in Table 6.1 demonstrates that *T. timopheevii* might also possess considerable useful genetic variability for micronutrients which can be used for wheat improvement.

*T. timopheevii* possess useful genetic variability and is resistant to many fungal diseases and pests (e.g. leaf rust resistance) as discussed in chapter 3. It will be highly appropriate to identify variability of *T. timopheevii* for high micronutrient content for introgression and pyramiding of the desired variability to achieve high levels of essential micronutrient content in wheat.

#### **5.1.7 Aims and objectives of this chapter:**

The main aims of this chapter are:

- 1) To determine the useful variability for micronutrient concentration in seeds of different accessions of *T. timopheevii*
- 2) To determine the relationship between seed and leaf micronutrient content of *T. timopheevii* accession P95-99.1-1
- 3) Analyse the micronutrient concentrations between *T. timopheevii* accession P95-99.1-1 in crosses to wheat and in different generations through the backcross generations.

## **5.2 Material and methods**

### **5.2.1 Plant Material**

The experimental material comprising of seven wild accessions of *T. timopheevii* and bread wheat (*T. aestivum*) cultivar Paragon, maintained at the WRC (Wheat research centre) seed store at University of Nottingham. The collection sites, their subspecies and accession number for all the wild accessions used in this study have been already given in Chapter 3, material and methods, section 3.2.1.

964 backcross individuals (24 F<sub>1</sub>, 63 BC<sub>2</sub>, 51 BC<sub>2</sub>F<sub>1</sub>, 432 BC<sub>2</sub>F<sub>2</sub>, 190 BC<sub>3</sub>, 86 BC<sub>3</sub>F<sub>1</sub>, 118 BC<sub>4</sub>) were used for leaf mineral analysis come from the crosses made between wheat cultivar (variety Paragon) and *T. timopheevii* accession P95-99.1-1. The detailed crossing scheme and their maintenance have been already given in Chapter 3, section 3.2.2.

### **5.2.2 Grain analysis**

Whole grain samples (dry seeds) of each accession of *T. timopheevii* and *T. aestivum* (var. Paragon) were ground (usually three to four seeds) and digested as per the standard procedure described by Thomas *et al.* (2016). Digestion, analysis of samples by ICP-MS was done in the same way as described in Chapter 2, section 2.8 for leaf samples. Concentration of minerals was expressed in mg/kg on dry weight basis.

### **5.2.3 Leaf analysis**

Leaves from Paragon and donor parent (*T. timopheevii* ssp. *timopheevii* accession P95-99.1-1) and their backcrosses were collected between ear emergence and before anthesis. One fully expanded leaf was removed from the tops of the stems of each plant. Leaf samples were dried in paper bags at 50°C for 48h and hand crushed to a powder within the bag, and 0.1g was subsampled for mineral analysis. SPAD meter (SPAD-502, Minolta, Japan) reading to

measure the chlorophyll content was done on the same leaves taken for mineral analysis. Digestion, analysis by ICP-MS of leaf samples has already been discussed in Chapter 2, material and methods, section 2.8. Concentration of minerals was expressed in mg/kg on dry weight basis. Only one replication of backcross individuals was possible as the seeds were in the segregating populations and differed for the introgression segments from *T. timopheevii*.

#### **5.2.4 Data processing**

For each data point, element-specific operational blank concentrations (ICP-run means) were subtracted. Data was then multiplied by initial sample volume, divided by the initial dry mass of plant material, and converted to mg element kg<sup>-1</sup> of dry leaf or seed material.

#### **5.2.5 Statistical Analysis**

All the graphs were plotted and analysed in PRISM software (ver. 7.03) and SigmaPlot13 software (SYSTAT Software). Using PRISM software data was analysed by analysis of variance (ANOVA) followed by F test at P<0.05 and correlation coefficient.

### **5.3 Results**

The bread wheat cultivars for commercial cultivation possess low levels of essential mineral nutrient content with very low variability, thus emphasizing the necessity of their biofortification for high essential mineral content. The genetic diversity for mineral nutrient concentration in seeds was studied in a collection of seven wild *Triticum timopheevii* accessions (P95-99.1-1, 538429, 427998, 355452, 427414, 538512 and 289752) and domesticated bread wheat (var. Paragon) (Figure 5.1).

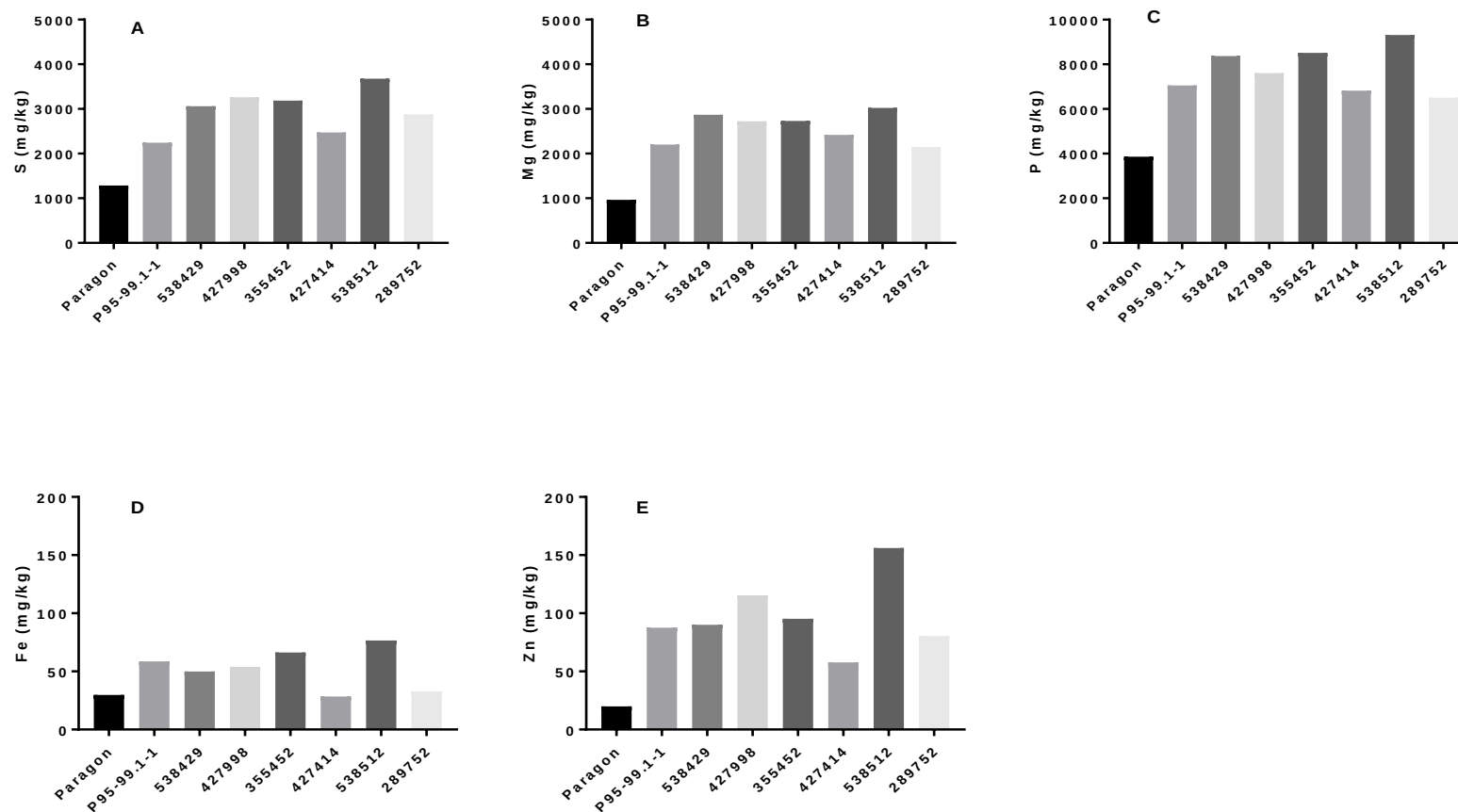


**Figure 5.1:** Mineral seed data for Paragon and different *T. timopheevii* accessions represented on the X axis, showing variation for A) Sulphur (S), B) Magnesium (Mg), C) Phosphorus (P), D) Iron (Fe) and E) Zinc (Zn) in mg kg<sup>-1</sup>, respectively.

The highest values of Sulphur (S), Magnesium (Mg), Phosphorus (P), Iron (Fe) and Zinc (Zn) concentrations in the seven wild accessions of *T. timopheevii* (P95-99.1-1, 538429, 427998, 355452, 427414, 538512 and 289752) were almost two-fold greater than the respective values in the wheat (var. Paragon) as shown in the Table 5.2. Among all the accessions of *T. timopheevii* studied, accession 538512 showed the highest concentrations of Mg (3024.73 mg/kg), S (3678.32 mg/kg), P (9314.95 mg/kg), Fe (76.53 mg/kg) and Zn (156.14 mg/kg) as compared to wheat (var. Paragon) Mg (966.46 mg/kg), S (1284.66 mg/kg), P (3864.89 mg/kg), Fe (29.77 mg/kg) and Zn (19.94 mg/kg) respectively.

**Table 5.2:** Different concentrations of Mg, S, P, Fe and Zn in mg/kg for Paragon and seven wild accessions of *T. timopheevii*.

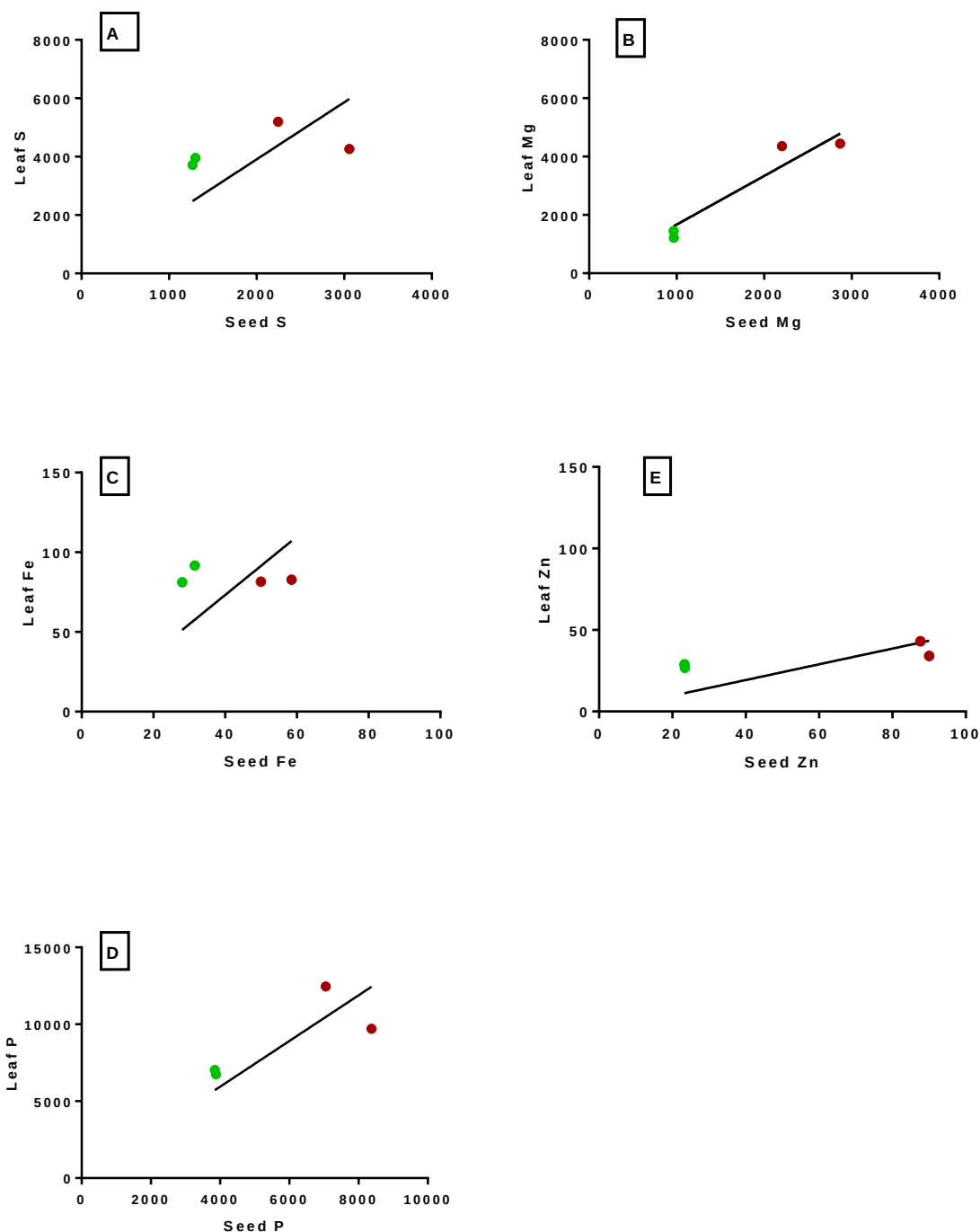
| <b>Genotypes</b>  | <b>Mg<br/>(mg/kg)</b> | <b>S<br/>(mg/kg)</b> | <b>P<br/>(mg/kg)</b> | <b>Fe<br/>(mg/kg)</b> | <b>Zn<br/>(mg/kg)</b> |
|-------------------|-----------------------|----------------------|----------------------|-----------------------|-----------------------|
| <b>Paragon</b>    | 966.46                | 1284.66              | 3864.89              | 29.77                 | 19.94                 |
| <b>P95-99.1-1</b> | 2202.27               | 2245.4               | 7055.43              | 58.54                 | 87.63                 |
| <b>538429</b>     | 2866.71               | 3058.48              | 8374.64              | 49.96                 | 90.01                 |
| <b>427998</b>     | 2721.44               | 3258.69              | 7602.36              | 53.88                 | 115.39                |
| <b>355452</b>     | 2730.12               | 3185.88              | 8512.85              | 66.28                 | 95.22                 |
| <b>427414</b>     | 2418.21               | 2472.82              | 6824.54              | 28.39                 | 57.81                 |
| <b>538512</b>     | 3024.73               | 3678.32              | 9314.95              | 76.53                 | 156.14                |
| <b>289752</b>     | 2141.79               | 2877.26              | 6500.29              | 32.74                 | 80.44                 |

**Table 5.3:** One-way anova analysis of seed content of Mg, S, P, Fe and Zn between Paragon and *T. timopheevii* accessions, P value (<0.05).

|                 |         |
|-----------------|---------|
| P value         | <0.0001 |
| P value summary | ****    |
| R square        | 0.9128  |

A comparison between the wild *T. timopheevii* accessions and the wheat cultivar Paragon by one-way anova for S, Mg, P, Fe and Zn was carried out (Table 5.3). There were highly significant differences observed between the wheat (var. Paragon) and *T. timopheevii* accessions for S, Mg, P, Fe and Zn seed content. All the seven wild accessions of *T. timopheevii* tested, exhibited a significantly positive correlation ( $P < 0.0001$ ,  $R^2 = 0.9128$ ) between wild accessions of *T. timopheevii* and wheat (var. Paragon) for S, Mg, P, Fe and Zn respectively (Table 5.3).

Among all the seven *T. timopheevii* accessions, accession P95-99.1-1 was used for the majority of the backcrossing programme. To confirm whether the accession of *T. timopheevii* ssp. *timopheevii*, accession P95-99.1-1 used for crossing with high mineral content in the grains also had higher content in leaves, different mineral content in the leaves (taken before anthesis) of parents [(*T. timopheevii*, P95-99.1-1 and wheat (var. Paragon))] were analysed (Figure 5.2).



● *T. timopheevii*  
 ● Paragon

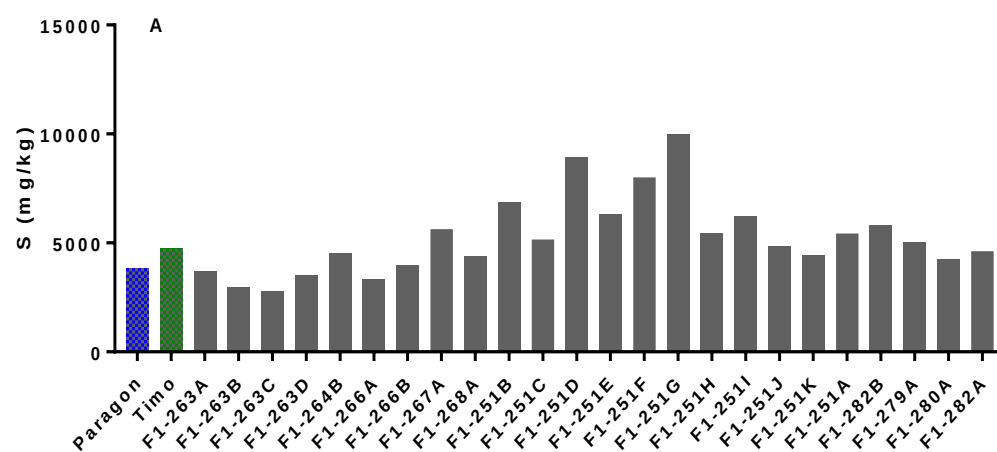
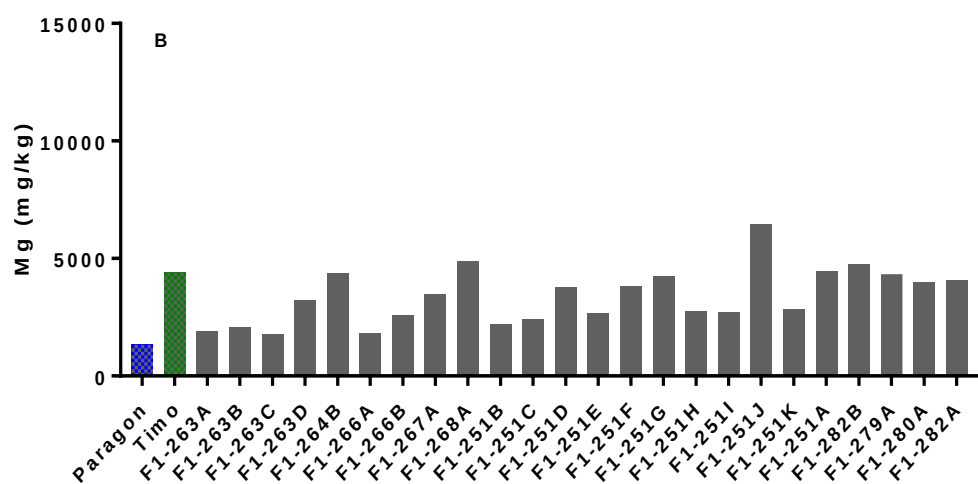
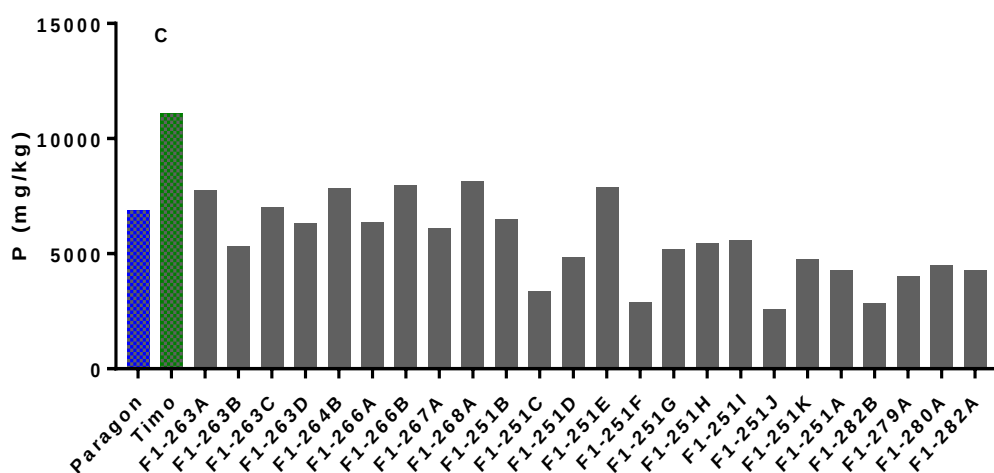
**Figure 5.2:** Relationship between seed and leaf for wheat (var. Paragon) and *T. timopheevii* accession P95-99.1-1 for A) Sulphur (S), *P* value: 0.013, B) Magnesium (Mg), *P* value: 0.001 C) Phosphorus (P), *P* value: 0.003 D) Iron (Fe) *P* value: 0.013 and E) Zinc (Zn), *P* value: 0.023, in mg kg<sup>-1</sup>, respectively. The line is regression line. The red solid circles (leaves) and the squares (seed) represent *T. timopheevii* and the green solid circle (leaves) and square (seed) represents wheat (var. Paragon).



Significant positive correlations were drawn between leaf and grain (A) S ( $P$  value: 0.0129,  $r=0.51$ ), (B) Mg ( $P$  value: 0.0010,  $r=0.59$ ), (C) P ( $P$  value: 0.0035,  $r=0.67$ ), (D) Fe ( $P$  value: 0.0130,  $r=0.54$ ) and (E) Zn ( $P$  value: 0.0235,  $r=2.07$ ) content for *T. timopheevii* accession P95-99.1-1 analysed (Figure 5.2). This indicates that the accession with high mineral content in grains also had higher mineral contents in their leaves.

The mineral contents of leaves of sterile  $F_1$  interspecific hybrids which were produced by crossing *T. timopheevii* (acc. P95-99.1-1) as the male parent with wheat (var. Paragon, *Ph1*) as the female parent, was also analysed and compared with their wheat (var. Paragon) and *T. timopheevii* parent for some of the hybrids. A total of 24  $F_1$  interspecific hybrids were analysed and compared with wheat (var. Paragon) and *T. timopheevii* parents (Figure 5.3).

Legends to follow after the end of all the graphs below. The X axis of the graphs represents Paragon, *T. timopheevii* (Timo) and  $F_1$  hybrids.



Graphs continued next page

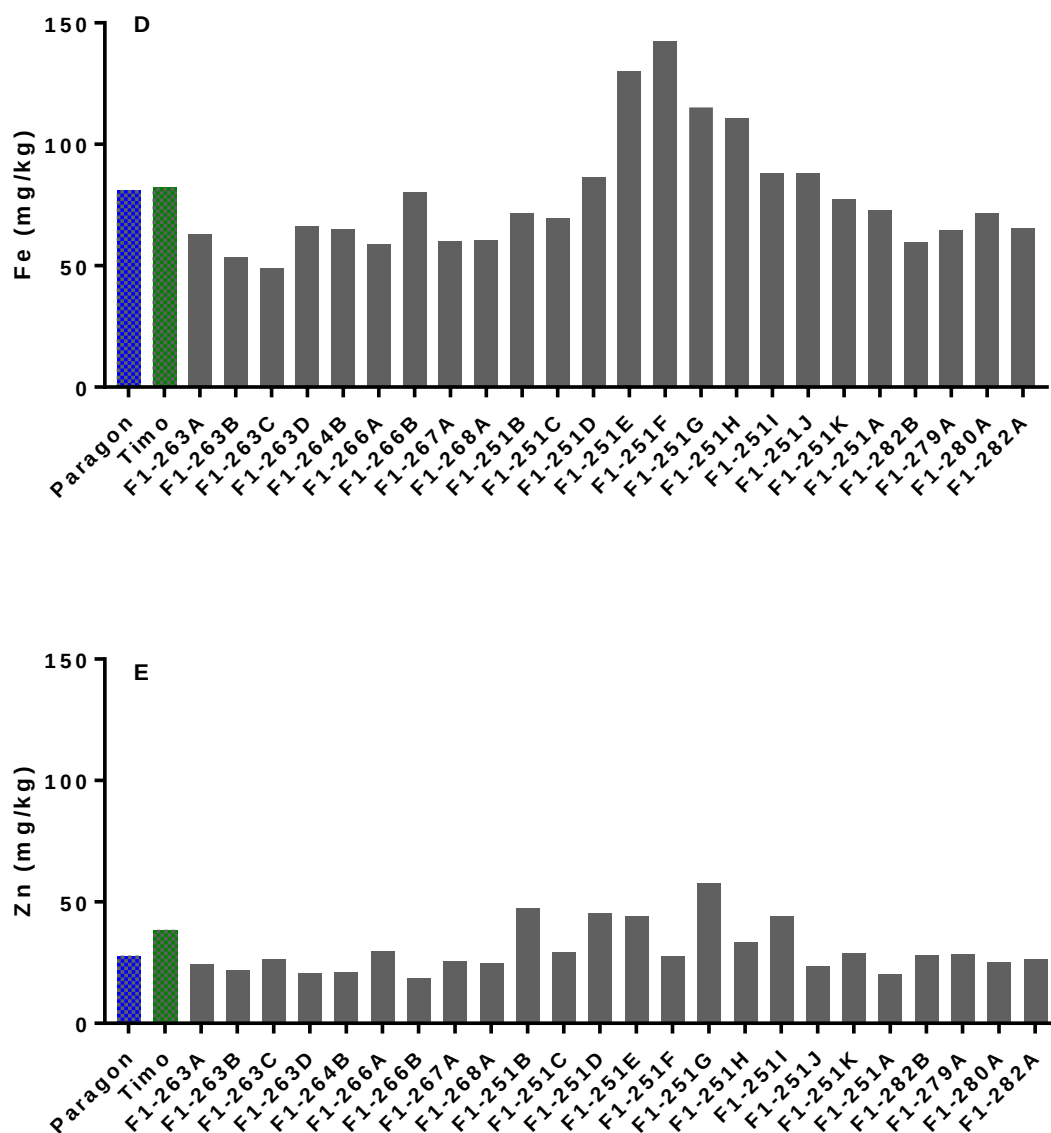
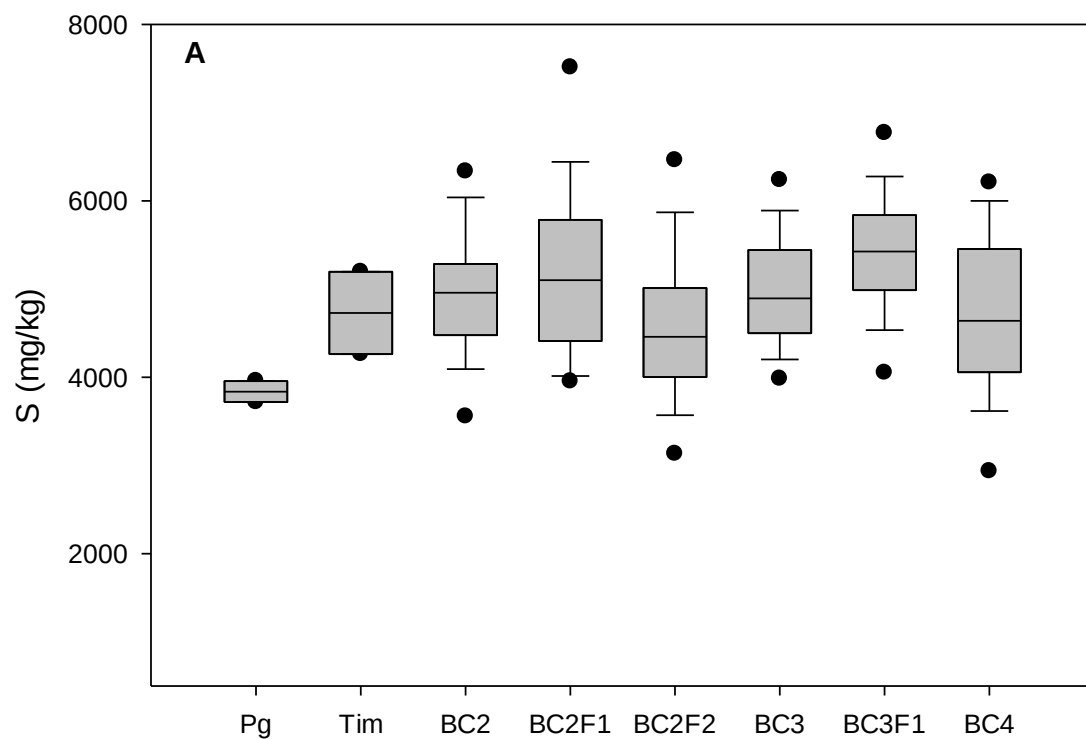
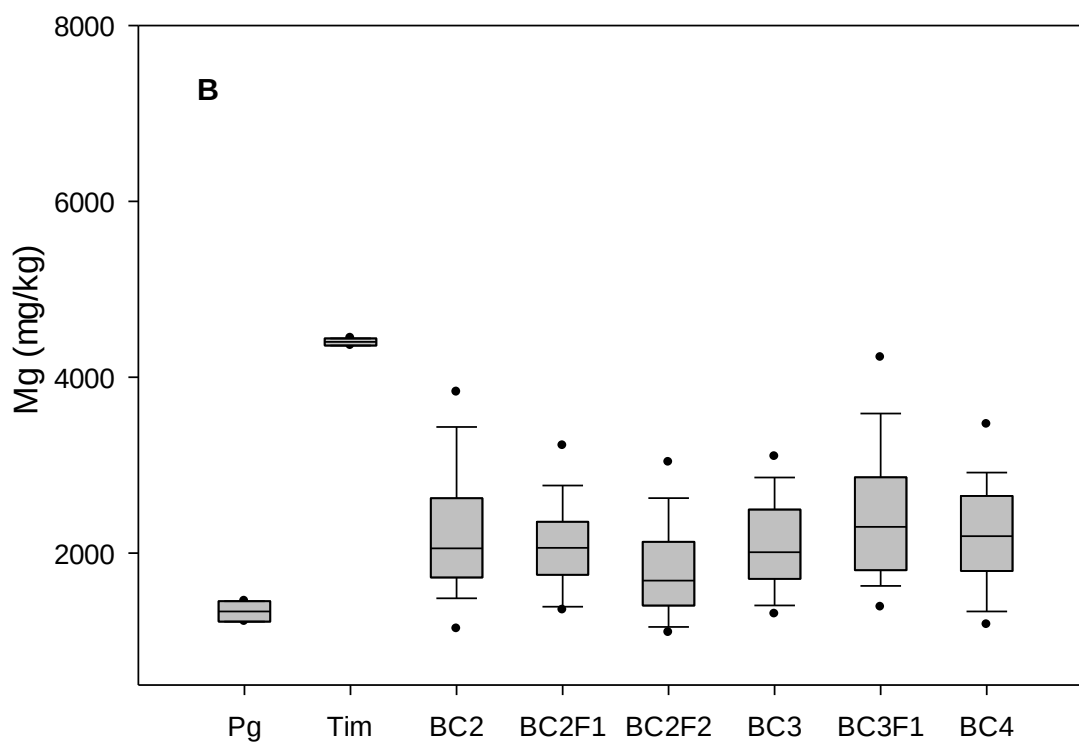


**Figure 5.3:** Different mineral A) Sulphur (S), B) Magnesium (Mg), C) Phosphorus (P), D) Iron (Fe) and E) Zinc (Zn) content in leaves of wheat cultivar (var. Paragon), *T. timopheevii* (acc. P95-99.1-1) parent and their F<sub>1</sub> interspecific hybrids.

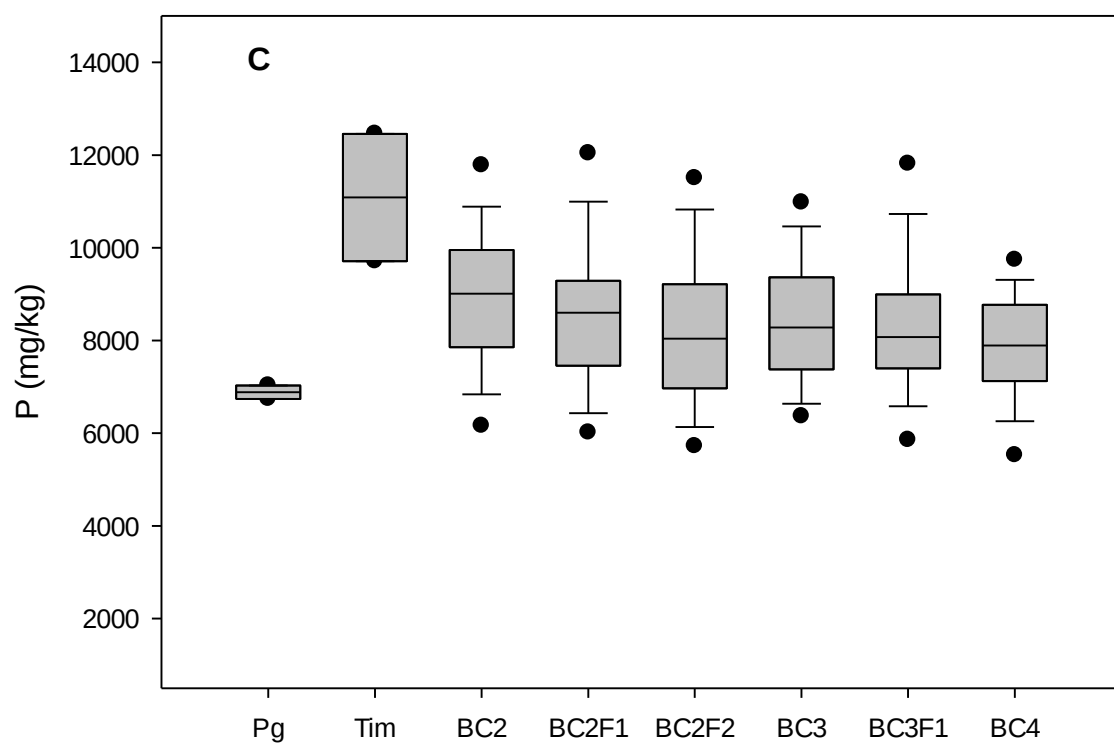
The leaves of some of the  $F_1$  hybrids had higher mineral contents than their wheat (var. Paragon) and *T. timopheevii* parent, whereas some hybrids had lower mineral contents than either of the parents (Figure 5.3). Mineral concentration of Phosphorus in particular for all the hybrids had lower content compared to *T. timopheevii* but variable (both high and low) content compared to wheat (var. Paragon). Leaves of some of the hybrids exceeded the level of mineral content in the *T. timopheevii* parent. Out of 24  $F_1$  interspecific hybrid analysed, 11 ( $F_1$ -267A,  $F_1$ -251B,  $F_1$ -251C,  $F_1$ -251D,  $F_1$ -251E,  $F_1$ -251F,  $F_1$ -251G,  $F_1$ -251H,  $F_1$ -251I,  $F_1$ -251A and  $F_1$ -282B) showed higher S content, 3 ( $F_1$ -268A,  $F_1$ -251J and  $F_1$ -282B) showed higher Mg content, 7 ( $F_1$ -251D,  $F_1$ -251E,  $F_1$ -251F,  $F_1$ -251G,  $F_1$ -251H,  $F_1$ -251I and  $F_1$ -251J) showed higher Fe content and 5 ( $F_1$ -251B,  $F_1$ -251D,  $F_1$ -251E,  $F_1$ -251G and  $F_1$ -251I) showed higher Zn content in their leaves as compared to *T. timopheevii* parent.

The sterile  $F_1$  hybrids between wheat and *T. timopheevii* were extensively backcrossed with the recurrent wheat parent to get backcross seed up to the fourth generation and alongside selfed to get  $BC_2F_1$ ,  $BC_2F_2$ ,  $BC_3F_1$  generations (see chapter 3 for germplasm production). A positive and significant correlation between leaf and grain mineral content was observed and also due to low number of backcross and self-seeds and every single seed being potentially unique with respect to the introgressions present in them (i.e. absence of homozygous seeds or segregating populations), digestion of leaves was done. Along with some of the backcross lines, some of the heads were self-fertile giving rise to self-seed were also analysed and compared to their parents (*T. timopheevii* and wheat (var. Paragon) during this study (Figure 5.4).

Legends to follow after the end of all the graphs below. The X axis of the graphs represent Paragon (Pg), *T. timopheevii* (Tim), (BC) for backcrossed lines.



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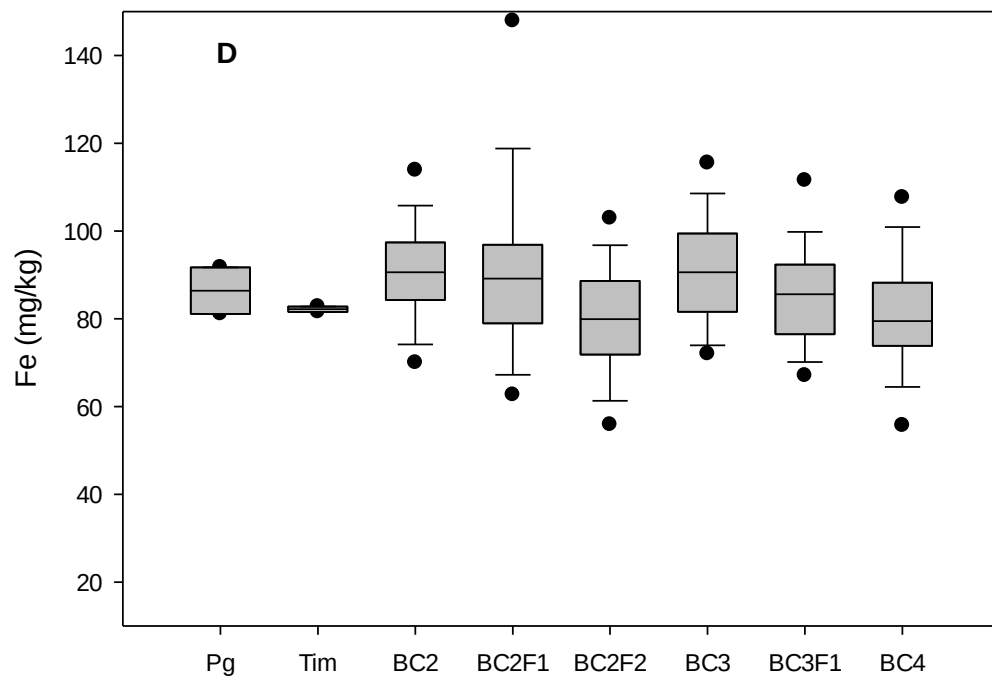
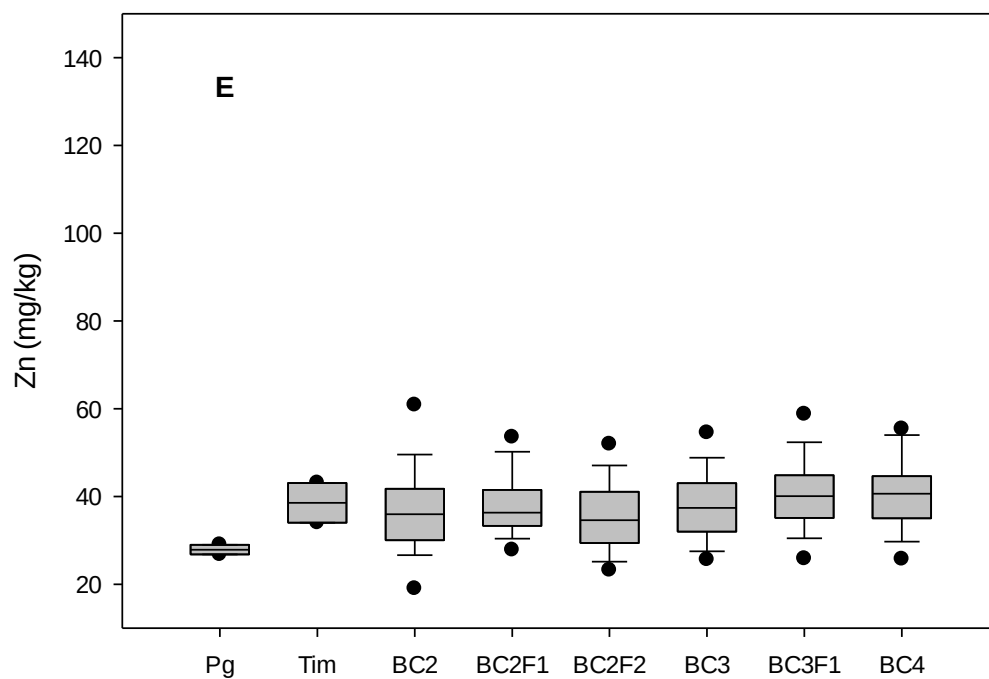
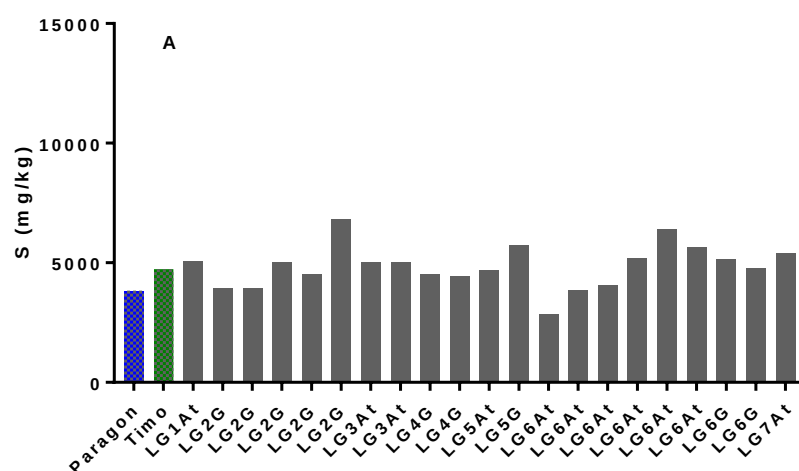
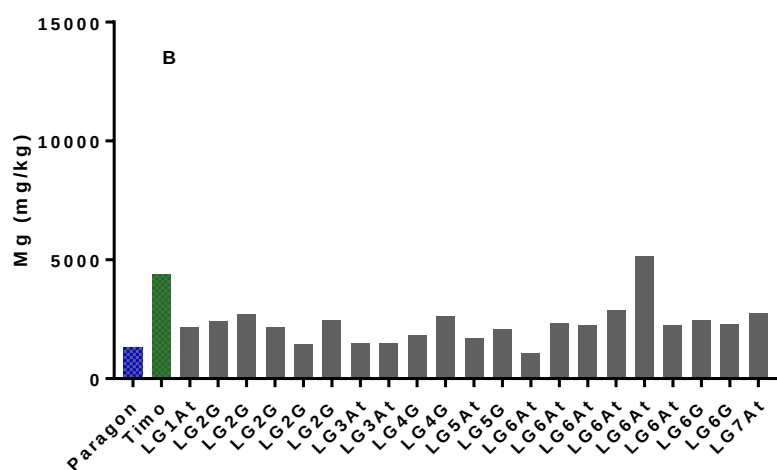
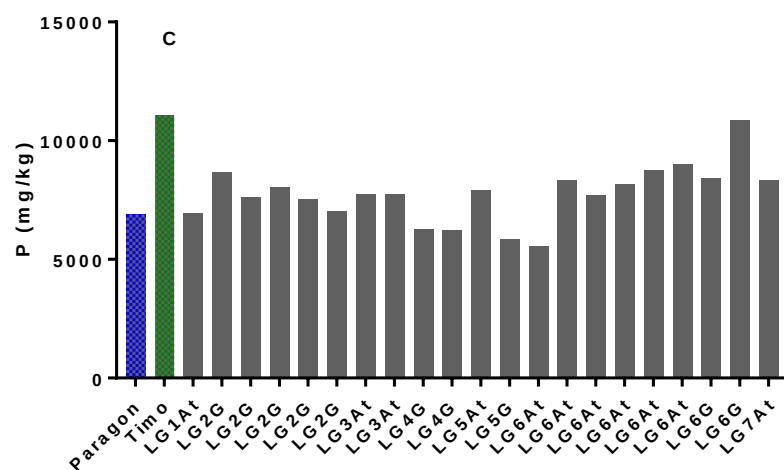


**Figure 5.4:** Variation between different backcrosses (BC<sub>2</sub>, BC<sub>3</sub> and BC<sub>4</sub>) and selfed generations (BC<sub>2</sub>F<sub>1</sub>, BC<sub>2</sub>F<sub>2</sub> and BC<sub>3</sub>F<sub>1</sub>) of *T. timopheevii* for A) Sulphur B) Magnesium C) Phosphorus D) Iron and E) Zinc in comparison to their parents [*T. timopheevii* and wheat (var. Paragon)].

Again 50% of the backcross lines with variable number of chromosomes and segments from *T. timopheevii* parent demonstrated higher and variable mineral contents (A) S, (B) Mg, (C) P, (D) Fe and (E) Zn, in their leaves compared to the wild donor parent (*T. timopheevii*) and wheat (var. Paragon) (Figure 5.4). Concentration of S in backcross and selfed lines was either higher or lower (4400-5500 mg/kg) than *T. timopheevii* (4800 mg/kg) but higher than wheat (var. Paragon) (3900 mg/kg). For Mg backcross and selfed lines concentrations were lower (1800-2800 mg/kg) than *T. timopheevii* (4200 mg/kg) and higher than wheat (var. Paragon) (1500 mg/kg). Similarly for P the backcross and selfed line concentration were lower (7500-9000 mg/kg) than *T. timopheevii* (1100 mg/kg) and higher than wheat (var. Paragon) (7000 mg/kg). Concentration of iron in backcross and selfed lines was either higher or lower (70-90 mg/kg) than *T. timopheevii* (82 mg/kg) and wheat (var. Paragon) (83 mg/kg). Concentration of Zn in backcross and selfed lines was again either higher or lower (27-40 mg/kg) than *T. timopheevii* (39 mg/kg) but higher than wheat (var. Paragon) (24 mg/kg) respectively. Hence leaves of both backcross lines and self-seed showed a larger variation for mineral content than both of the parents [*T. timopheevii* and wheat (var. Paragon)].

Every single individual backcross seed also accounted to different number of segments or linkage groups (see Chapter 4 for genotyping) present from *T. timopheevii* as the whole genome transfer approach was followed in the present study. Thus to observe the effect of presence of different linkage groups on the mineral content, 21 backcross plants with single introgression from different linkage groups were examined for each of the minerals under study (Figure 5.5). Legends to follow after the end of all the graphs below. The X axis of the graphs represents Paragon, *T. timopheevii* (Timo) and the linkage groups (LG).





Graphs to be continued in next page

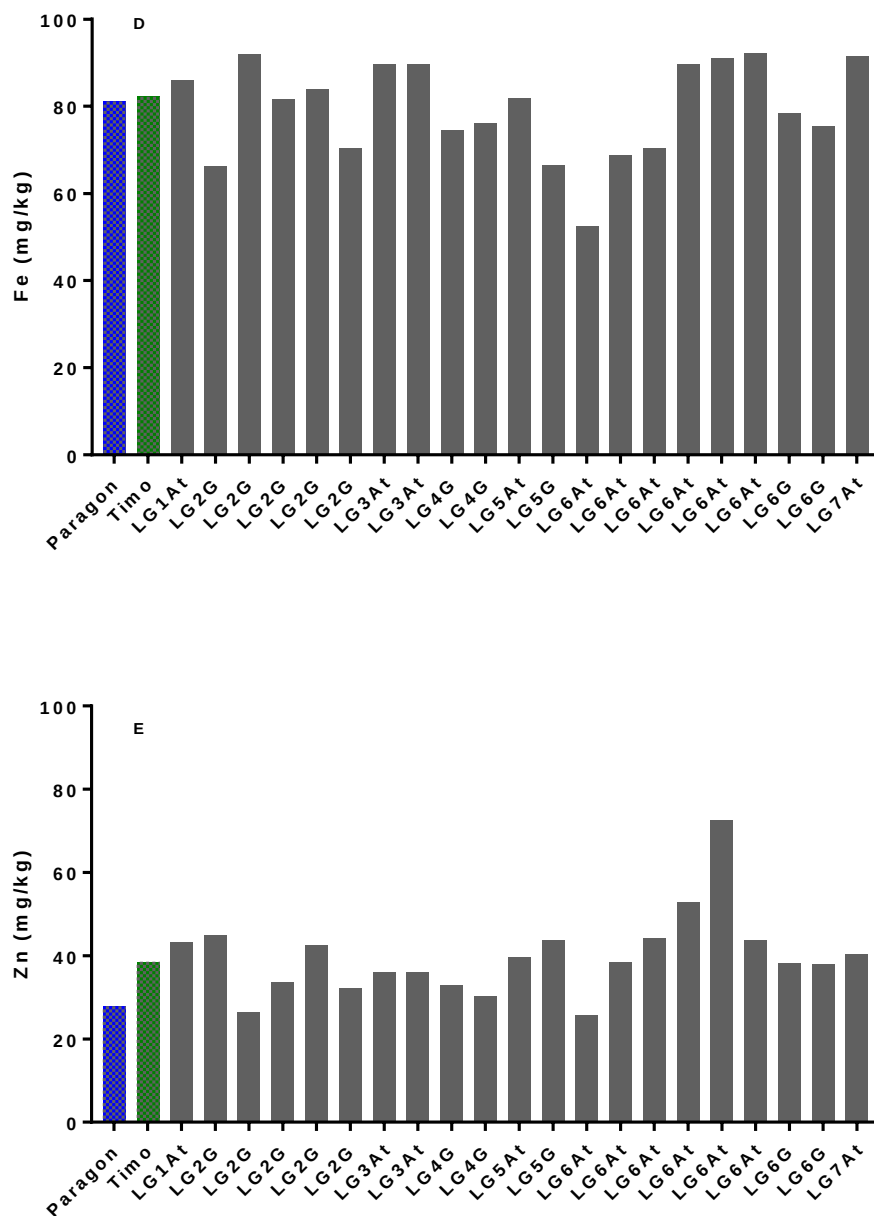


**Figure 5.5:** Leaf data of different single introgression lines for A) Sulphur (S) B) Magnesium (Mg) C) Phosphorus (P) D) Iron (Fe) E) Zinc (Zn) with different linkage groups.

Variation in mineral content (A) S, (B) Mg, (C) P, (D) Fe and (E) Zn, in majority of the backcross/introgression lines with different linkage group was observed compared to the wild parent *T. timopheevii* and wheat (var. Paragon) (Figure 5.5).

The chlorophyll contents are supposed to be directly related to the presence of magnesium levels (Tanoi and Kobayashi, 2015). To see the relationship between magnesium and chlorophyll contents of the leaves, the SPAD measurements were taken on the leaves that were selected for digestion before anthesis and were plotted against the content of magnesium for the respective backcross lines and parents [*T. timopheevii* and wheat (var. Paragon)] (Figure 5.6).

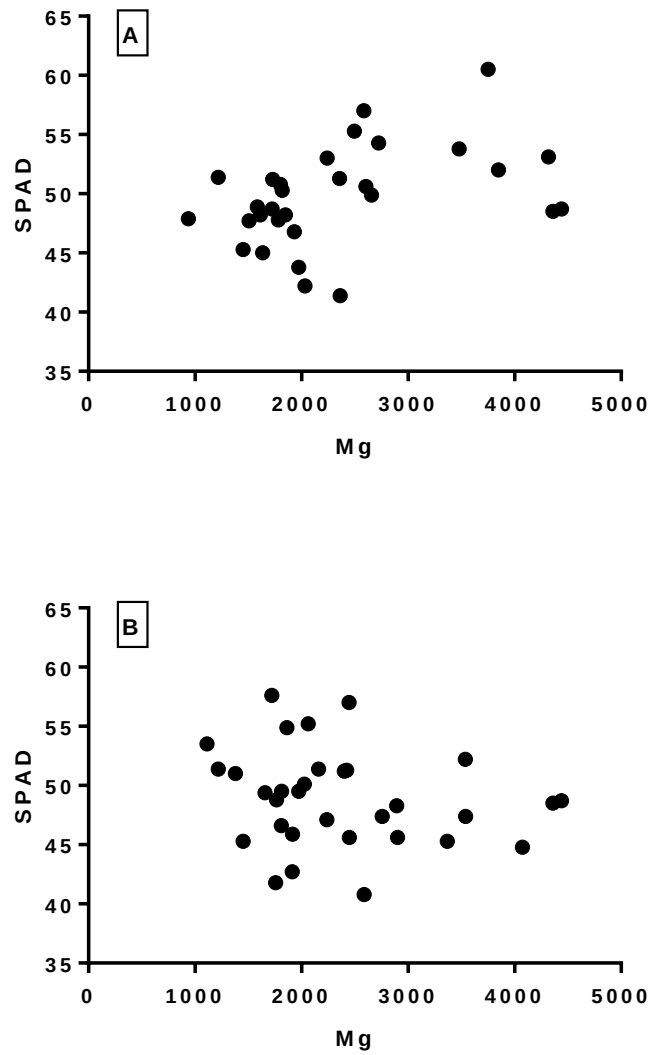


**Figure 5.6:** Relationship between magnesium ( $\text{mg/kg}^{-1}$ ) and chlorophyll levels (SPAD values) in (A) between parents [*T. timopheevii* and wheat (var. Paragon)] and backcross plants (18 BC<sub>2</sub> and 8 BC<sub>3</sub> and (B) between parents [*T. timopheevii* and wheat (var. Paragon)] and backcross plants (12 BC<sub>2</sub>, 8 BC<sub>2</sub>F<sub>1</sub>, and 8 BC<sub>3</sub>), respectively. The line is the regression line.

Significant positive correlation ( $P < 0.0001$ ) between Mg and SPAD values for the backcross lines and parents [*T. timopheevii* and wheat (var. Paragon)] was observed (Figure 5.6).

## 5.4 Discussion

Most of the high yielding bread and durum wheat cultivars have low mineral contents (Cakmak *et al.*, 2000), whereas the related wild Triticum and Aegilops species possess useful variability for high mineral concentration (Chuuneja *et al.*, 2006; Rawat *et al.*, 2009; Ardalani *et al.*, 2016). Screening of a number of accessions of tetraploid wild *T. timopheevii* species has shown that the A<sup>t</sup> and G genomes may be the useful source for transferring genes controlling high mineral content to wheat. The G genome being closely related to the B genome of polyploid wheat (Kostoff, 1936; Love, 1941) can be effectively used for transferring useful variability for high mineral content into wheat. *T. monococcum*/*T. urartu* with A<sup>m</sup> genome most closely related to that of the A genome of polyploid wheat, is supposed to be the donor of A<sup>t</sup> genome of *T. timopheevii* and have already been reported to have high grain iron and zinc content (Monasterio and Graham, 2000; Cakmak *et al.*, 2000). Ozkan *et al.* (2007) observed a wide range of variation for grain iron, zinc, magnesium and copper among 54 accessions of *T. monococcum*. Thus, the useful variability from the A<sup>t</sup> and G genomes can be transferred into wheat through induced homoeologous chromosome pairing using *ph1* (Chen *et al.*, 1994).

Leaves of *T. timopheevii* ssp. *timopheevii*, accession P95-99.1-1 showed higher micronutrient content than wheat (var. Paragon) for S, Mg, P, Fe and Zn mg/kg respectively (Figure 2). Significant high positive correlation between grain and leaf content of these minerals in donor (*T. timopheevii*) strongly suggests that presence of a genetic component in the donor for more efficient uptake/transport of the micronutrients as compared to the bread wheat (var. Paragon). Rawat *et al.* (2009) also found significant relationship between leaf

and grain iron and zinc in *Aegilops* accessions. This positive correlation between leaf and grain minerals suggests the possibility of using leaf instead of grain for an early selection of plants with potentially high minerals in the segregating generations of interspecific crosses rather than waiting till harvest for grain analysis. This could facilitate selection of high yielding and disease resistant plants among those plants with high minerals in leaves similar to that of marker assisted selection.

Further higher and variable mineral content in leaves of most of the  $F_1$  interspecific hybrids than their parents provides a concept that the screened wild accession of *T. timopheevii* P95-99.1-1 has the requisite superior genetic system(s) for higher uptake/transport of micronutrients content in leaves which could ultimately be mobilized to grains. Higher mineral content in majority of the hybrid lines suggests that the superior genetic system of *T. timopheevii* is partially due to dominant gene(s) and is capable of expression in association or in the background of cultivated wheat and hence could be targeted and exploited. These results indicated that *T. timopheevii* ssp. *timopheevii* might be a promising genetic resource with high S, Mg, P, Fe and Zn concentrations for the biofortification of current wheat cultivars. This is supported by Hu *et al.*, (2017) where all the 12 accessions of *T. timopheevii* ssp. *timopheevii* showed higher concentrations of grain and flag leaf Fe and Zn than Chinese Spring (CS) and durum wheat cultivar Langdon (LDN).

Backcross individuals ( $BC_2$ ,  $BC_3$ ,  $BC_4$ ) along with self-seeds ( $BC_2F_1$ ,  $BC_2F_2$ ,  $BC_3F_1$ ) in their advanced backcross generations showed variation ranging from that of the wheat parent to their wild donor. This variation indicates the synergistic interaction between parents and the possibility of obtaining transgressive segregants for high minerals. Further variation in mineral content in

introgression lines with different linkage groups compared to their respective parents suggests that the variation could be attributed to the presence/transfer of one or more chromosomes/chromosome segments of the wild donor, capable of expression in association i.e. dominant gene(s) responsible for the superior genetic system of *T. timopheevii* had a cumulative effect and not subjected to particular linkage group, controlling the efficient uptake and translocation of the micronutrients.

A significant positive correlation between Mg and chlorophyll content in backcross lines and their donor parents proves the hypothesis that magnesium values are directly related to chlorophyll content of the leaves of the plant. Hence, suggesting the possibility of using leaf chlorophyll content for an early selection of plants with potentially high magnesium levels, in the backcross generations.

The lines containing whole additions, substitution, and introgressed chromosome segments developed with *T. timopheevii* using the whole genome transfer approach, may not be very suitable at this stage for commercial exploitation due to instability of the genomes as they are still segregating populations and due to linkage drag as well, also it's a preliminary data due to lack of replication as the experiment was not designed in particular to study mineral concentrations. Once the lines are made homozygous, transfer of defined chromosomal regions containing genes controlling high mineral concentration and content should be followed for the future work using induction homoeologous chromosome pairing. Those improved lines/cultivars or varieties could make an important contribution to improving the health of millions of people worldwide to avoid micronutrient malnutrition which still affects over 25% (particularly for Iron and Zinc) of the global population.

## **Chapter 6**

### **Screening of *T. timopheevii* for Fusarium head blight resistance**

#### **6.1 Introduction**

Fusarium head blight (FHB) also known as Scab is a highly destructive and important disease of all classes of wheat worldwide that causes significant yield and quality losses. The FHB species complex comprises of more than 16 species (O' Donnell *et al.*, 2004), that infect a range of hosts (van der Lee *et al.*, 2015). It includes *Fusarium avenaceum* (Fries) saccardo, *F. crookwellense*, *F. graminearum*, *F. culmorum*, *F. langsethiae* Torp and Nirenberg, *F. Poae* (Peck) Wollenweber, *F. sporotrichioides* Sherbakoff, *Microdochiumnivale* and *M. majus* (Fr.) Samuels and I.C. Hallett (Dill-Macky, 2010). The disease is primarily caused by the predominant species, *F. graminearum* Schwabe (Schroeder and Christensen, 1963). The causal pathogen, *F. graminearum* can cause the accumulation of harmful mycotoxins in the grain. Mycotoxins are low molecular weight metabolites produced by fungi (Desjardins and Hohn, 1997). Toxins are chemically and thermally stable and can be found in raw grain and finished products. The most common mycotoxins are deoxynivalenol (DON), 3-acetyldeoxynivalenol (3ADON), 15-acetyldeoxynivalenol (15ADON), nivalenol (NIV) and its acetylated derivative 4-acetyl-nivalenol (4ANIV) (Desjardins *et al.*, 2006). Among all the FHB mycotoxins, 15-DON is the dominant FHB chemotype globally (van der Lee *et al.*, 2015).

The plant tissues infected by Fusarium species also produce another type of mycotoxins known as 'Masked mycotoxins'. In order to prevent xenobiotic effects, plants convert the chemical structure of mycotoxins as a defence mechanism (Galaverna *et al.*, 2009). For example, plants can convert the



Fusarium toxins DON and ZEA by glucosyltransferase enzymes into deoxynivalenol-3-glucoside and zearalenone-14-glucoside, respectively, which are not virulence factors (Berthiller *et al.*, 2013, 2015). Masked mycotoxins or conjugated mycotoxins can cause a health hazard, if toxins are released during digestion (Rychlik *et al.*, 2014). No threshold level for masked DON has been set by the European Commission. This can lead to a health hazard if DON-3-glucoside can pass the radar undetected and cause serious health consequences for human and animals (Panel, 2014).

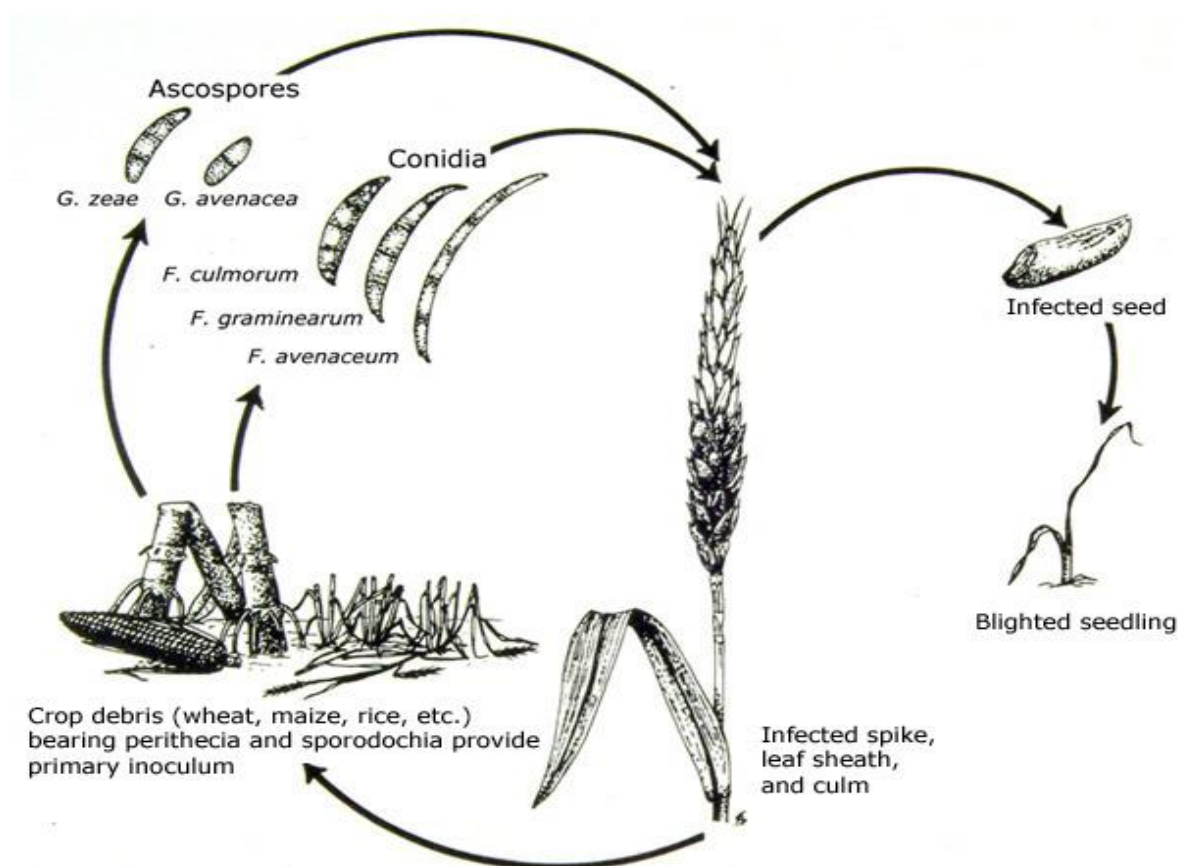
FHB occurs in a number of important wheat growing regions; North and South America (Walker *et al.*, 2001), Europe (Yli-Mattila *et al.*, 2009), Australia (Mcknight and Hart, 1966), Canada (Fernando *et al.*, 2006), Kenya (Boutigny, 2011) and South Africa (van Wyk *et al.*, 1988). Wheat is susceptible to infection from the flowering (anthesis) stage through to the soft dough stage of kernel development. Once infected, FHB causes premature bleaching of spikes starting with one or more spikelets on a spike, which proceeds until the whole spike is whitened. These bleached spikes are sterile containing lightweight and shrivelled kernels which are chalky white or pink in appearance. These scabby kernels are also called tombstones because of their dull and lifeless appearance (McMullen *et al.*, 1997). These infected kernels are also referred to as Fusarium-damaged kernels (FDK) which ultimately affects market price. Infected kernels contain mycotoxins, primarily deoxynivalenol (DON) which is also called vomitoxin and is an emetic (Gale, 2003), resulting in poor weight gain in ruminants. In non-ruminant animals like pigs, ingestion of infected kernels results in feed refusal (Desjardins, 2006).

Epidemics of this disease result in significant economic losses, and both the quality and quantity of wheat grains is compromised. FHB was first described as a major threat to wheat in England in 1884 (Goswami and Kistler, 2004). Since then, numerous epidemics have been reported worldwide: outbreaks occurred in the 1900s in North America with losses of up to \$4.8 billion. Indirect losses are seen in poor quality food products, reduced productivity of livestock, and costs incurred for treatment of both humans and animals (Wegulo, 2012). Further, in the northern Great Plains, the total economic loss due to FHB has been estimated at \$6.2 billion from 1993 through 2001 (Nganje *et al.*, 2004).

#### **6.1.1 Life cycle**

The long term survival of *F. graminearum* is significant, making the control of *F. graminearum* even more difficult. FHB can survive as a saprophyte on crop debris from a previous season or on weeds, other plants, seeds and insects (Parry *et al.*, 1995; Champeil *et al.*, 2004). FHB is predominantly caused by infection with wind borne ascospores produced from the sexual stage and splash borne macroconidia produced from the anamorph stage (Figure 6.1) (Parry *et al.*, 1995; Bai and Shaner, 1994 and Sutton, 1982). Infection of the wheat spikes are caused through water splash or wind containing ascospore or macroconidia. In warm temperatures and high humidity ascospores are prone to germination forming perithecia. From 16°C a perithecia can form and as the temperature increases to about 25°C, the amount of perithecia also increase, resulting in even more ascospores available to infect wheat heads.

**Figure 6.1:** Life cycle of FHB



Sutton (1982)

### 6.1.2 Resistant types

FHB resistance is quantitative, generally being controlled by several to many genes of medium to minor effect. Resistance is broadly classified into two forms, morphological and physiological (Gilsinger *et al.*, 2005; Loeffler *et al.*, 2009; Buerstmayr *et al.*, 2009 and Liu *et al.*, 2009). Type I resistance is the first hurdle to infection by Fusarium species and may conform to the definition of morphological resistance. It is resistance to infection and is measured as disease incidence. Type I resistance quantitative trait loci (QTLs) in the FHB-resistant germplasm 'Wangshuibai', include *Qfhi.nau-5A* on chromosome 5A and *Qfhi.nau-4B* on chromosome 4B (Xue *et al.*, 2010, 2011). Physiological resistance also called active resistance, is the most studied form of resistance to FHB in wheat

and has been classified into five types (Mesterhazy, 1995). Type II resistance is the second form of resistance that functions against fungal spread from the infection point within spikes and have been reported in varieties such as 'Sumai 3' (Browne, 2009). Type II resistances are generally classified as physiological resistance. The QTLs responsible for type II resistance on chromosomes 3BS and 6BL in 'Sumai 3' have been characterised and defined as single genes termed *Fhb1* and *Fhb2*. A recent report claims to have cloned *Fhb1* (Rawat *et al.*, 2016) although other data suggest that the situation is more complex (P. Nicholson, pers. comm.). Type III resistance is the prevention of DON accumulation within spike (Miller *et al.*, 1985). Type IV prevention to kernel infection and type V is the prevention of yield loss. Type IV and V resistance definitions are rarely used due to the difficulty in understanding the resistance mechanisms (Mesterhazy, 1995). Type I and Type II are the resistant types that are generally being used in evaluating material within breeding programmes. Type III resistance is the third frequently used quantitative method where DON concentrations in grains are being measured (Bai *et al.*, 2001).

The passive resistance mechanism include plant height (dwarfness increase while tallness decrease natural infections), presence of awns (awns increase the disease severity while the absence of awns decreases the disease severity), spikelet density within the head (loose ears have a lower natural infection than dense ones), and escape by flowering at the booting stage (Cook, 1981).

### **6.1.3 Inoculation techniques**

Inoculation is the deliberate introduction of a pathogen to the host. By inoculation the effect of the host-pathogen interaction can be studied to determine the resistance response. This interaction is important to assess the

disease resistance in new pre-breeding lines. This concept led to the development of different inoculation techniques to test and screen pre-breeding material for FHB resistance. Spray and point inoculation are the two most frequent used methods (Mesterhazy, 1995).

- 1) **Point inoculation:** Point inoculation is the injection of a spore suspension via a pipette to a single floret (Schroeder and Christensen, 1963; Engle *et al.*, 2003). By using a pipette, equal amounts of inoculum can be injected, preventing invalid results. The volume injected varies between 5-20  $\mu$ l and the spore concentration is based on pathogenicity of the *Fusarium* spp. (McCallum and Tekauz, 2002). Point inoculation is more labour intensive and time-consuming compared to spray inoculation. Point inoculation is mainly used to evaluate type II resistance (resistance to pathogen spread) (Miedaner *et al.*, 2003). The stable nature of type II resistance makes it more favourable to evaluate FHB in wheat breeding (Burlakoti *et al.*, 2010; Bai and Shaner, 1994). Point inoculation is also the method of choice for glasshouse trials (Miedaner *et al.*, 2003). After the spore suspension is injected into a single floret, the injected wheat plant is placed in a dew chamber for a period of 72h post-inoculation. Bagging the head is also an important step to create controlled humidity (Mesterhazy *et al.*, 1999).
- 2) **Spray inoculation-** Spray inoculation is the physical spray of a spore suspension onto the head at the anthesis stage (Miedaner *et al.*, 2003). It can be used to assess type I and II resistance. It is less labour intensive compared with point inoculation when a large field experiment is evaluated. It resembles the natural host-pathogen interaction. The

inoculum suspension can be sprayed more than once and the concentration can differ based on *Fusarium* spp. (Dill-Macky *et al.*, 2003). *F. culmorum* and *F. graminearum* are the two species that are highly pathogenic on small-grain cereals and so are most commonly used in disease trials (Edwards, 2004).

- 3) **Grain spawn**- This is another method used for the screening of large amounts of material in the field. In this method pre-infected wheat, oat or maize grains serve as the source of inoculum and the grain is spread throughout the field to allow perithecia to develop and release ascospores that infect the crop. Disease assessment is followed in the same way as spray inoculation.

No single disease management strategy has eliminated the risk of FHB. Therefore, an integrated approach that begins with selecting the best available genetic resistance coupled with timely application of fungicide to control FHB, particularly when the environment favours disease occurrence is preferred. Development and deployment of disease resistant varieties is possibly the most economical and efficient control strategy. Wheat breeders worldwide are trying to contain FHB through resistance breeding strategies. Breeding for varieties with high levels of resistance combined with good quality and yield is extremely difficult because much resistance derives from poorly adapted varieties which causes linkage drag when introduced into high yielding commercial varieties. Resistance is affected by environmental conditions, which makes it difficult to score disease severity where varieties are in favour of infection at the flowering stage and grain fill (Dill-Macky, 2010). Because scab resistance is limited to a

few varieties, genetic resources must be broadened through the use of the wheat gene pool.

#### **6.1.4 FHB resistance from wild relative source**

Identification of novel sources of resistance is vital for the development of wheat cultivars with robust and durable resistance to this devastating disease. Wild relatives of wheat have proven to be a rich gene source for wheat improvement including the genes for FHB resistance. The wild species mostly evaluated in the past had a perennial growth habit and were collected from geographical areas with a climate favourable for epidemics of FHB. It was postulated by Wan *et al.*, (1997a) that the FHB resistance was accumulated in these accessions by gene mutations and natural selection under high disease pressure. Different wild species and genera have been targeted for the transfer of FHB resistance to common wheat which includes *T. dicoccoides*, *T. timopheevii*, *T. monococcum*, *Ae. speltoides*, Synthetic hexaploid wheat lines, Roegneria, Hystrix, Elymus, Agropyron etc. (Fedak *et al.*, 2004, 2017; Cai *et al.*, 2005).

In the evaluation of accession of *T. monococcum*, *Ae. speltoides*, *T. timopheevii*, *Ae. cylindrica*, Triticale (wheat-rye amphiploid), Tritordeum (*Hordeum chilense* X Durum wheat, amphiploid) (Fedak *et al.*, 1997, 2004, 2015, 2017) and accessions of Thinopyrum species such as *Th. elongatum*, *Th. junceiforme*, *Th. intermedium*, *Th. ponticum*, *Th. junceum* (Jauhar and Peterson, 1998, 2001; Cai *et al.*, 2005) type II resistance for FHB was found. Whereas, both type I and type II resistance was found in genus Elymus (Mujeeb-Kazi *et al.*, 1983; Wang *et al.*, 1986, 1991; Wan *et al.*, 1997b; Ban, 1997), where *E. Humidus* further exhibited FHB resistance at a level higher than 'Sumai 3' (Cai *et al.*, 2005) (Table 6.1).

**Table 6.1:** Screening of various wild relatives of wheat for FHB resistance and the QTLs mapped on different chromosomes.

| Species                                                       | Screening                | Cross                                    | Population produced                                | Type of resistance | Significance                                   | Reference                                                 |
|---------------------------------------------------------------|--------------------------|------------------------------------------|----------------------------------------------------|--------------------|------------------------------------------------|-----------------------------------------------------------|
| <i>T. monococcum</i>                                          | 200 accession screened   | M321 X AC Domain wheat (Spring cultivar) | 80 DH lines                                        | Type II            | QTL on chromosome 5A linked to <i>Xwme</i> 705 | Fedak <i>et al.</i> , 1997, 2000, 2003, 2011              |
| <i>Ae. speltooides</i>                                        | 50 accession screened    | S184 X AC Domain                         | F <sub>1</sub> hybrid                              | Type II            | Lowered DON content                            | Fedak <i>et al.</i> , 1997, 2004, 2015                    |
| <i>T. timopheevii</i>                                         | 1 accession              | TC67 X AC Brio                           | F <sub>7</sub> population of 230 RIL               | Type II            | QTL mapped to chromosome 5A                    | Cao <i>et al.</i> , 2009; Malihipour <i>et al.</i> , 2016 |
| <i>Ae. cylindrica</i>                                         | 1 accession              | Cy1-1 X AC Superb, AC Barrie and Alsen   | F <sub>4</sub> , F <sub>6</sub> and F <sub>7</sub> | Type II            | Odessa 129-2 with low DON content              | Fedak <i>et al.</i> , 1997                                |
| Triticale (wheat X rye)                                       | 371 strains of Triticale | TMP16315 X AC Ultima                     | 150 DH lines                                       | Type II            | Susceptible                                    | Fedak <i>et al.</i> , 1997                                |
| <i>T. miguschovae</i> ( <i>T. timo</i> X <i>T. tauschii</i> ) | Amphiploid               | Amphiploid X AC Superb                   | 35 BC <sub>2</sub> F <sub>5</sub>                  | Type II            | MSB55 with low DON content                     | Davoyan and Ternovskaya, 1996; Zhironov, 1980             |
| Wild emmer wheat                                              | 151 accession screened   |                                          |                                                    | Type II            | 8 resistant accession                          | Buerstmayr <i>et al.</i> , 2003                           |
| <i>Tritordeum</i>                                             | 7 Amphiploid             | Amphiploid X Capelli                     | 17 BC <sub>3</sub> F <sub>4</sub>                  | Type II            | All 17 with low DON                            | Fedak <i>et al.</i> , 2017                                |



|                                                                                                                                                                                                                             |                        |                  |  |                    |                                                                                                                                   |                                                                   |
|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------|------------------|--|--------------------|-----------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------|
| ( <i>Hordeum chilense</i> X Durum cultivar Ma)                                                                                                                                                                              | evaluated              | <i>Ph</i> mutant |  |                    | content                                                                                                                           |                                                                   |
| Wild emmer wheat ( <i>T. turgidum</i> subsp. <i>dicoccoides</i> )                                                                                                                                                           | 282 accession screened |                  |  | Type II            | Found 10 resistant accession                                                                                                      | Miller <i>et al.</i> , 1998                                       |
| Wild emmer wheat                                                                                                                                                                                                            | 416 accession screened |                  |  | Type II            | Wide variation in response to FHB                                                                                                 | Oliver <i>et al.</i> , 2007                                       |
| <i>T. turgidum</i>                                                                                                                                                                                                          | 376 accession screened |                  |  | Type II            | 16 accessions of <i>T. turgidum</i> subsp. <i>carthlicum</i> and 4 <i>T. turgidum</i> subsp. <i>dicoccum</i> accessions resistant | Oliver <i>et al.</i> , 2008                                       |
| <i>Elymus giganteus</i> L. (syn. <i>Leymus racemosus</i> subsp. <i>racemosus</i> ), <i>Roegneria kamoji</i> and <i>Agropyron kamoji</i> and <i>Roegneria ciliaris</i> (syn. <i>Elymus ciliaris</i> subsp. <i>ciliaris</i> ) | Accessions screened    |                  |  | Type I and Type II | Highly resistant to FHB                                                                                                           | Mujeeb-Kazi <i>et al.</i> , 1983; Wang <i>et al.</i> , 1986, 1991 |
| 13 <i>Roegneria</i>                                                                                                                                                                                                         | 17 accessions          |                  |  | Type I and Type II | 31 accessions with high levels of Type I                                                                                          | Wan <i>et al.</i> , 1997b                                         |

|                                                                                                                                             |                                  |  |  |                    |                                                                                         |                                 |
|---------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------|--|--|--------------------|-----------------------------------------------------------------------------------------|---------------------------------|
|                                                                                                                                             |                                  |  |  |                    | and II resistance                                                                       |                                 |
| Elymus genus                                                                                                                                | Four indigenous Japanese species |  |  | Type I and Type II | <i>E. humidus</i> and <i>E. racemifer</i> highly resistant to FHB                       | Ban, 1997                       |
| Tetraploid wheat species including Persian wheat, wild emmer wheat, cultivated emmer wheat, Polish wheat, Oriental wheat, and Poulard wheat | 255 accessions                   |  |  | Type II            | One resistant accessions of Persian wheat and four accessions of cultivated emmer wheat | Cai <i>et al.</i> , 2005        |
| <i>Th. elongatum</i> (syn. <i>Lophopyrum elongatum</i> ) and <i>Th. junceiforme</i>                                                         | Accessions screened              |  |  | Type II            | Both resistant                                                                          | Jauhar and Peterson, 1998, 2001 |

Even though no complete resistance or immunity to FHB has been observed to date, genotypic variation is large in wild relatives of wheat. Overall in the screening of the various wild relatives of wheat the most resistance found to date has been Type II resistance, as it appears to be more stable and less affected by non genetic factors (Bai and Shaner, 1994). Type I resistance is more difficult to evaluate and therefore fewer reports have been published on cultivars possessing type I resistance. Therefore, wild relatives with type I resistance will be novel and useful for the wheat breeding programs.

For identification of novel sources of resistance genes for FHB, many wild relatives of wheat have been hybridized with wheat, resulting in the production of numerous amphiploids, addition, substitution, and translocation lines and synthetic hexaploid lines. Various examples of the attempts for transfer of FHB resistance from wild relatives of wheat into wheat is given in Table 6.2.

**Table 6.2:** Introgression of FHB resistance from wild relative source into wheat and the respective QTLs mapped for resistance.

| Species                                         | Population                                                                   | Method            | QTLs for Resistance                                                              | Reference                                               |
|-------------------------------------------------|------------------------------------------------------------------------------|-------------------|----------------------------------------------------------------------------------|---------------------------------------------------------|
| <i>L. racemosus</i>                             | Addition and substitution lines ( <i>T. aestivum</i> X <i>L. racemosus</i> ) | RFLP analysis     | Homoeologous groups 5 and 7 conferred resistance to FHB                          | Qi <i>et al.</i> , 1997                                 |
| <i>R. kamoji</i>                                | <i>T. aestivum</i> X <i>R. kamoji</i>                                        | RFLP analysis     | Homoeologous group 1 and 2                                                       | Wang <i>et al.</i> , 1999, 2001                         |
| <i>Hordeum chilense</i><br><i>Th. elongatum</i> | Chinese Spring background                                                    |                   | 1H <sup>ch</sup> of <i>Hordeum chilense</i><br>7E of <i>Th. elongatum</i>        | Fedak <i>et al.</i> , 2003<br>Shen <i>et al.</i> , 2004 |
| <i>T. dicoccoides</i>                           | Disomic substitution lines of (Langdon X <i>T. dicoccoides</i> )             |                   | Chromosome 5B and 7A of <i>T. dicoccoides</i>                                    | Stack <i>et al.</i> , 2003                              |
| <i>T. macha</i>                                 | 'Hobbit-sib' X 'Hobbit-sib' ( <i>T. macha</i> )                              | DH population     | QTL for Type I resistance from chromosome 4AS co-segregating with <i>Xgwm165</i> | Steed <i>et al.</i> , 2005                              |
| <i>L. elongatum</i>                             | Cultivated wheat X <i>L. elongatum</i>                                       | Molecular markers | FHB resistant gene on long arm of 7E and lines exhibited type II FHB resistance  | Shen <i>et al.</i> , 2004<br>Shen and Ohm, 2006         |
| <i>E. repens</i> (L.)<br>Gould                  | Introgression lines with cultivated wheat                                    | Molecular markers | Type II FHB resistance in eight lines                                            | Zeng <i>et al.</i> , 2013                               |
| <i>Remus</i>                                    | DH population (CM-82036 X <i>Remus</i> )                                     | Molecular markers | QTL mapped to chromosome 3BS and 5A                                              | Buerstmayr <i>et al.</i> , 2002, 2003b                  |
| Emmer wheat                                     | Emmer line FA-15-3                                                           |                   | FHB resistant QTL on 3AS                                                         | Stack <i>et al.</i> , 2002; Otto <i>et al.</i> , 2002   |

Overall, resistance to FHB is quantitatively inherited and the responsible chromosomal regions were identified on many chromosomes of wild relatives, Homoeologous group 5 and 7 of *L. racemosus* (Qi *et al.*, 1997), homoeologous group 1 and 2 of *R. kamoji* (Wang *et al.*, 1999, 2001), 1H<sup>ch</sup> chromosome of *Hordeum chilense* (Fedak *et al.*, 2003), 7E of *Th. elongatum* (Shen *et al.*, 2004), 5B and 7A of *T. dicoccoides* (Stack *et al.*, 2003), 4AS of *T. macha* (Steed *et al.*, 2005), 7EL of *Th. elongatum* (Shen *et al.*, 2004; Shen and Ohm, 2006), 3BS and 5A of *Remus* (Buerstmayr *et al.*, 2002, 2003b) and 3AS of emmer wheat (Stack *et al.*, 2002; Otto *et al.*, 2002).

Although QTLs from different sources of resistance have been mapped and in some cases successfully used in wheat breeding programs, finding new source of resistance is needed to avoid complete dependence on limited sources. Introgression of additional resistance genes and pyramiding FHB resistance QTLs in wheat lines may lead to development of wheat lines/cultivars with an enhanced level and stability of resistance to prevent economic damage under high disease pressure. *T. timopheevii* (PI 343447) is a source of FHB resistance which has been used to introgress resistance into wheat (Fedak *et al.*, 2004; Cao *et al.*, 2009). Recently Malihipour *et al.*, (2017) with the help of SSR markers, mapped a novel QTL on chromosome 5AL for type II resistance in a RIL population derived from the cross between 'AC Brio' (bread wheat cultivar) and 'TC 67' (FHB resistant cultivar derived from *T. timopheevii*). Mapping and tagging of FHB resistance available in wheat cultivars with *T. timopheevii* may be of great interest for use in wheat breeding programs.

### **6.1.5 Aims and objectives of this chapter**

The main aims of the work described in this chapter are:

- 1) Screening different accessions of *T. timopheevii* for type I and type II FHB resistance
- 2) Development of mapping populations between resistant and susceptible accessions of *T. timopheevii*
- 3) Study the associations between FHB resistance and morphological and developmental traits such as plant height, number of spikelets, rachis type, awn colour, stem colour and head colour

## **6.2 Material and methods**

### **6.2.1 Plant material**

Seven wild accessions of *T. timopheevii* maintained at WRC (wheat research centre) Nottingham, and the wheat variety Highbury were screened for type I and type II FHB resistance at John Innes centre (JIC) by Andrew Steed in 2015 and 2016. A FHB-resistant accession of *T. timopheevii* P95-99.1-1 was crossed to the susceptible accessions 538512 and 355452. For both crosses, P95-99.1-1 was taken as female parent and accessions 538512 and 355452 was taken as the male parent. The resulting F<sub>1</sub> plants were selfed to produce F<sub>2</sub> plants. A mapping population of 475 F<sub>2</sub> plants were established with the cross of P95-99.1-1 and 538512 and 4,128 F<sub>2</sub> plants were established with the cross of P95-99.1-1 and 355452. Out of 475, 128 F<sub>2</sub> plants from population 1 and 472 F<sub>2</sub> plants were germinated for the next round of screening and selfing. In total, 115 lines were selected from population 1 and 223 lines from population 2 and were evaluated for type I resistant reaction to FHB and agronomic traits in the polytunnel experiment 2017 at JIC.

### **6.2.2 Polytunnel trial 2015 and 2016**

Disease trials were conducted with seven accessions of *T. timopheevii* and wheat (var. Highbury) in a polytunnel at JIC. For point and spray trial of wild accession screening, a mixture of *F. graminearum* isolates (k1-4/k2-5) was used. Point inoculation used conidia at a concentration of  $1 \times 10^6$  injected into a single central spikelet per ear using a insulin syringe at mid-anthesis. Spray inoculation used conidia at a concentration of  $1 \times 10^5$  applied to single ears at mid-anthesis using a handheld sprayer. Both the methods enable evaluation for type II resistance and type I resistance, respectively. The trial was made up of 6 replicate blocks

containing 5-8 plants for each accession, including the Highbury wheat variety as a check. Disease was assessed several times by point inoculation and scored as the number of spikelets with disease symptoms above and below the point of inoculation at each time point. Following spray inoculation the heads were scored four times as the percentage of the infected spikelets to monitor disease progress.

### **6.2.3 Polytunnel trial 2017**

Disease trials were conducted with 338 lines of both mapping populations in a polythene horticultural tunnel to maintain humidity and temperature. The  $F_2$  material was grown in Nottingham and transferred to JIC for disease trial. For the spray trial of mapping populations in 2017 at JIC, a mixture of *F. culmorum* UK isolates FC2037/FC2076 was used. Temperatures in the polytunnel were slightly higher than ambient, with average daytime temperatures during June being 20°C, whereas light levels were slightly reduced because of the translucent polythene. Individual plants were grown in 1L pots filled with John Innes No. 2 loam-based potting compost adjusted to pH7.5 with lime. Two individual ears for each plant were spray-inoculated until run off, at mid-anthesis with  $1 \times 10^5$  conidia  $\text{ml}^{-1}$  inoculum and inoculation was repeated 4 days later. This method enabled evaluation for type I resistance. The inoculated ears were covered with a glassine bag 42 hours to maintain high humidity post inoculation and trickle irrigation was used to water the plants. Visual disease symptoms were evaluated 16 and 19 days post inoculation (dpi). Spray-inoculated ears were scored as number of the spikelets showing disease symptoms.



### Morphological traits

Measurements were taken for plant height, number of spikelets, rachis type, ear shape, awn colour, stem colour and head colour for each line of the two populations in the polytunnel trial by myself in 2017. Plant height was measured as the distance from the soil surface to the top of the head (excluding the awns). Rachis type was scored using a 0-3 scale; 0=flexible rachis, 1=less flexible rachis, 2=brittle rachis and 3=very brittle rachis this was later grouped into only two classes: brittle and non-brittle class (Figure 6.2). Ear shape was scored using a 1-4 scale: 1=lax, 2=moderately lax, 3=compact, 4=highly compact (Figure 6.3). Awn colour was scored using a scale of 1-3, where 1=light colour awns (normal), 2=brown colour awns and 3=black coloured awns (Figure 6.4). Stem colour was scored on 1-3 scale where 1=light colour (normal), 2=red colour stem and 3=red/purple colour stem (Figure 6.5). Head colour was scored using a 1-3 scale, where 1=light colour head (normal), 2=brown colour head and 3=black colour head (Figure 6.6).

**Figure 6.2:** Heads showing rachis type where a) represents the brittle rachis type and b) represents the non-brittle rachis type.



**Figure 6.3:** Heads showing four classes of ear morphology observed in two populations where 1) represents class 1, 2) represents class 2, 3) represents class 3 and 4) represents class 4.



**Figure 6.4:** Heads showing awn colour where a) represents the brown coloured awns, b) represents the colourless awns and c) represents the black coloured awns.





**Figure 6.5:** Genotypes showing the stem colour where a) represents the red/purple stem and b) represents the colourless stem type.



**Figure 6.6:** Head showing head colour where a) represents the brown coloured head, b) represents the colourless head and c) represents the black coloured head.



#### **6.2.4 Statistical analyses**

Disease mean for the different score dates was calculated and histograms for disease and various agronomic traits including scatter plots were generated using GenStat software (10<sup>th</sup> edition). Analysis of variance was carried out on visual disease scores for both populations. Pearson coefficients of correlation were used to analyse the relationship between coloured traits and disease scores and morphological trait means for both populations. Both ANOVA and correlation analysis were carried out using GraphPad PRISM software (ver. 7.03).

## 6.3 Results

### 6.3.1 Polyunnel trial 2015 and 2016

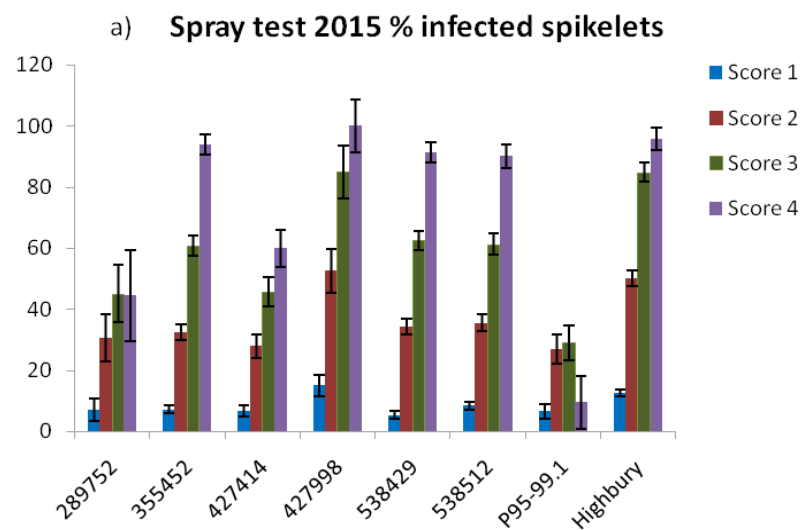
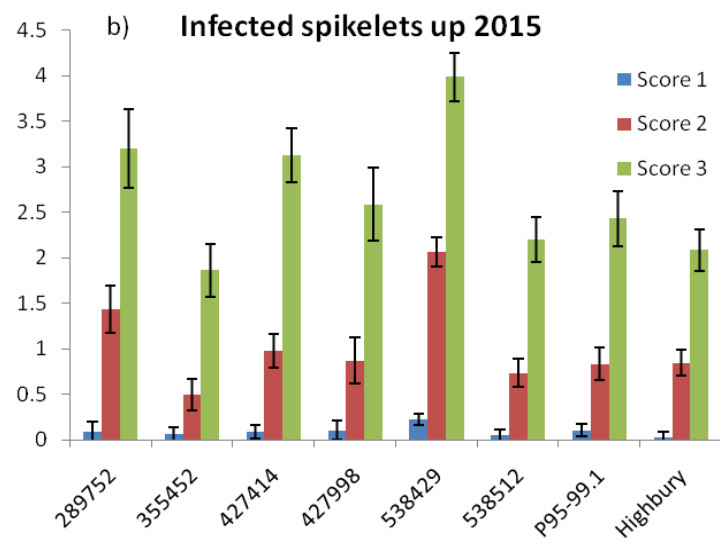
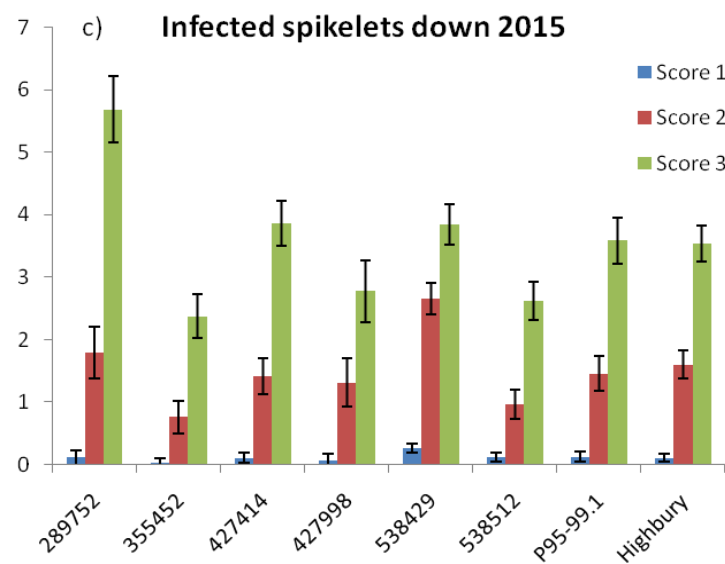
Two disease trials (2015 and 2016) for screening accessions of *T. timopheevii* were performed at JIC by Andrew Steed. FHB severity of all the accessions under study is presented as the percentage infection score for spray inoculation tests (Figure 6.7a and 6.7d) and as the number of infected spikelets with disease symptoms above and below the point of inoculation (Figure 6.7b, 6.7c, 6.7e and 6.7f). Following spray inoculation in 2015, the heads were scored four times to monitor disease progress. Overall infection scores, including those of accessions 355452, 427998, 538429 and 538512 along with Highbury were generally higher at score 4 (21d) than at score 3 (14d) post inoculation, indicating noticeable disease progress during the third week after inoculation (Figure 6.7a). Thus, the overall average FHB severity in these accessions was over 60% with ranges of 60% to 100%. The resistant accession P95-99.1-1, however exhibited consistently low levels of infection, with minimum disease development (less than 10%) at score 4 (21d) postinoculation than 20% at score 3 (14d) (Figure 6.7a). However, it should be noted that low level of disease at the higher score (score 4) could be due to error in scoring because of ripening of heads. The consistent resistance of accession P95-99.1-1 and the minimal disease development over time indicate the potency of this accession to combat this disease. Following point inoculation, almost all accessions of *T. timopheevii* exhibited severe disease symptoms with an overall average of 2 to 4 infected spikelets up and down each spike at 14d post-inoculation in the polyunnel (Figure 6.7b and 6.7c). Among them, accessions (289752, 427414 and 538429) showed high levels of susceptibility, exhibiting infection that spread to >50% of

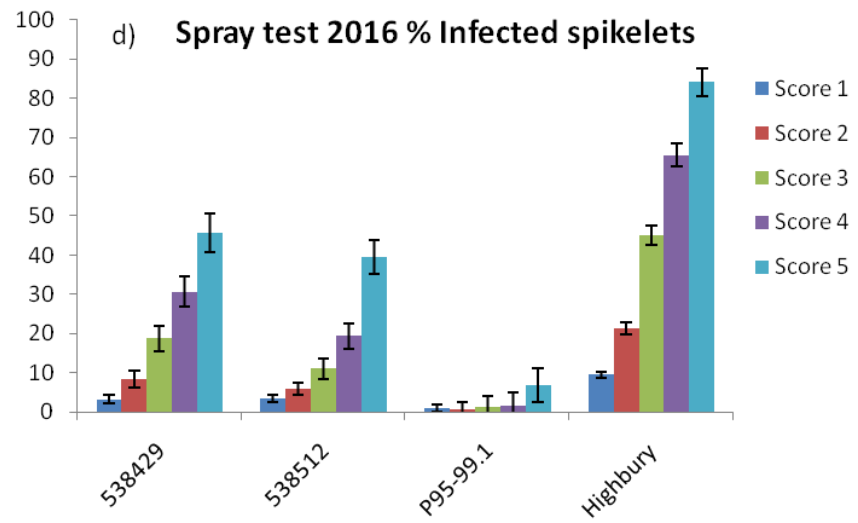
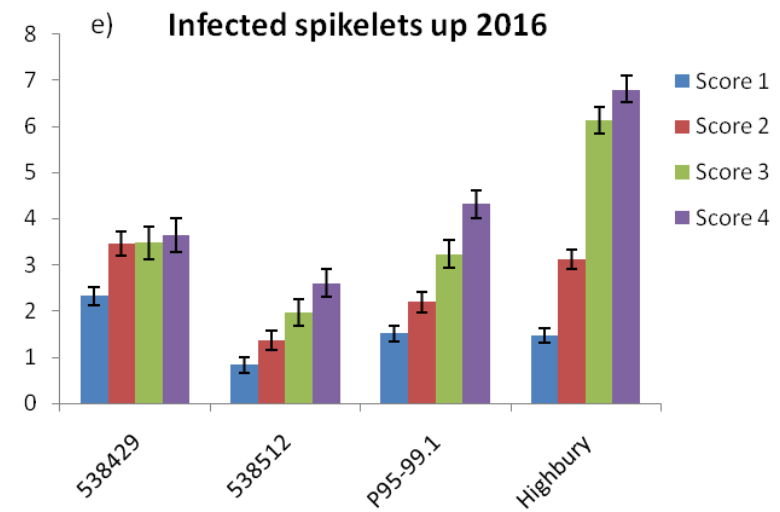
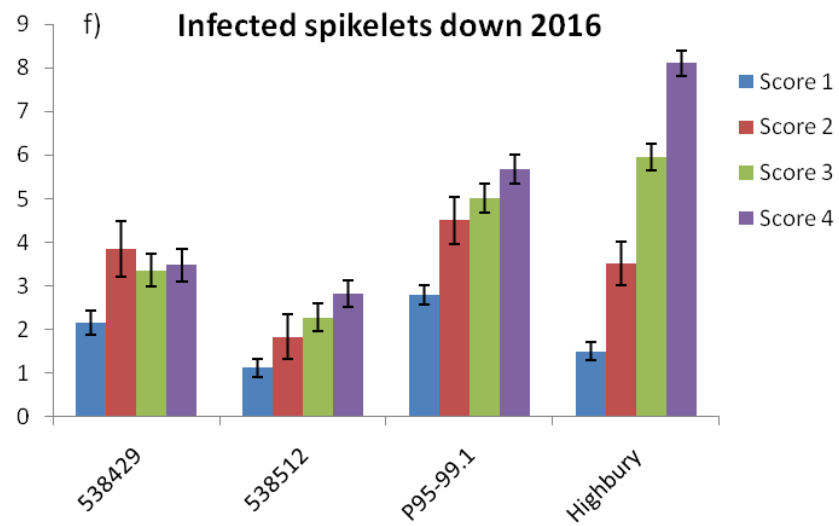


the spikelets in inoculated spikes. Strikingly, P95-99.1-1 was no more resistant than the other accessions indicating that it does not possess significant type II resistance.

To confirm the results of the 2015 trial, spray and point inoculation tests were conducted in 2016 with wheat cultivar Highbury and *T. timopheevii* accessions (538429, 538512 and P95-99.1-1) (Figure 6.7d, 6.7e and 6.7f). The spray test of 2016 confirmed the results of 2015, showing that accession P95-99.1-1 was the most resistant (less than 10% infection) to type I infection and both Highbury, 538429 and 538512 were more susceptible showing an average FHB severity over 40% (Figure 6.7d). Accession 538429 was the most susceptible out of the two (Figure 6.7d). The result for the type II point experiment indicates that Highbury and P5-99.1-1 were more susceptible for both spread up and down the spikes than 538429 and 538512 (Figure 6.7e and 6.7f).

**Figure 6.7:** Mean Fusarium head blight severity of a) seven accessions of *T. timopheevii* and Highbury following spray inoculation and b, c) point inoculation in 2015 trial and further in 2016 trial in Highbury, three accessions of *T. timopheevii* following d) spray inoculation and e, f) point inoculation. Disease is presented as percentage of infected spikelets (a and d) and number of infected spikelets (b, c, e and f). Error bars is equal to standard deviation.





Hence, the results obtained from both years trial following spray inoculation showed P95-99.1-1 to be highly resistant to infection while Highbury and other accessions were much more susceptible. Following point inoculation to access type II resistance almost all accessions exhibited severe disease symptoms. Accessions 289752, 427414 and 538429 were most susceptible in 2015 while P95-99.1-1 was the most susceptible accession in 2016. Overall, these trials revealed that P95-99.1-1 possesses a very high level of type I resistance (resistance to initial infection) while it lacks substantial type II resistance (resistance to spread within the head). On the basis of these results P95-99.1-1 was crossed to 538512 and 355452. A total of 6 crosses were made between P95-99.1-1 and 538512 giving rise to 40 F<sub>1</sub> hybrid seeds while a total of 3 crosses between P95-99.1-1 and 355452 gave rise to 44 F<sub>1</sub> hybrid seeds. Two mapping populations, population 1 (P95-99.1-1/538512) and population 2 (P95-99.1-1/355452) were further selfed to produce 475 and 4128 F<sub>2</sub> generations and out of these 115 from population 1 and 223 from population 2 were randomly chosen and studied for FHB resistance and various agronomic traits at JIC in 2017.

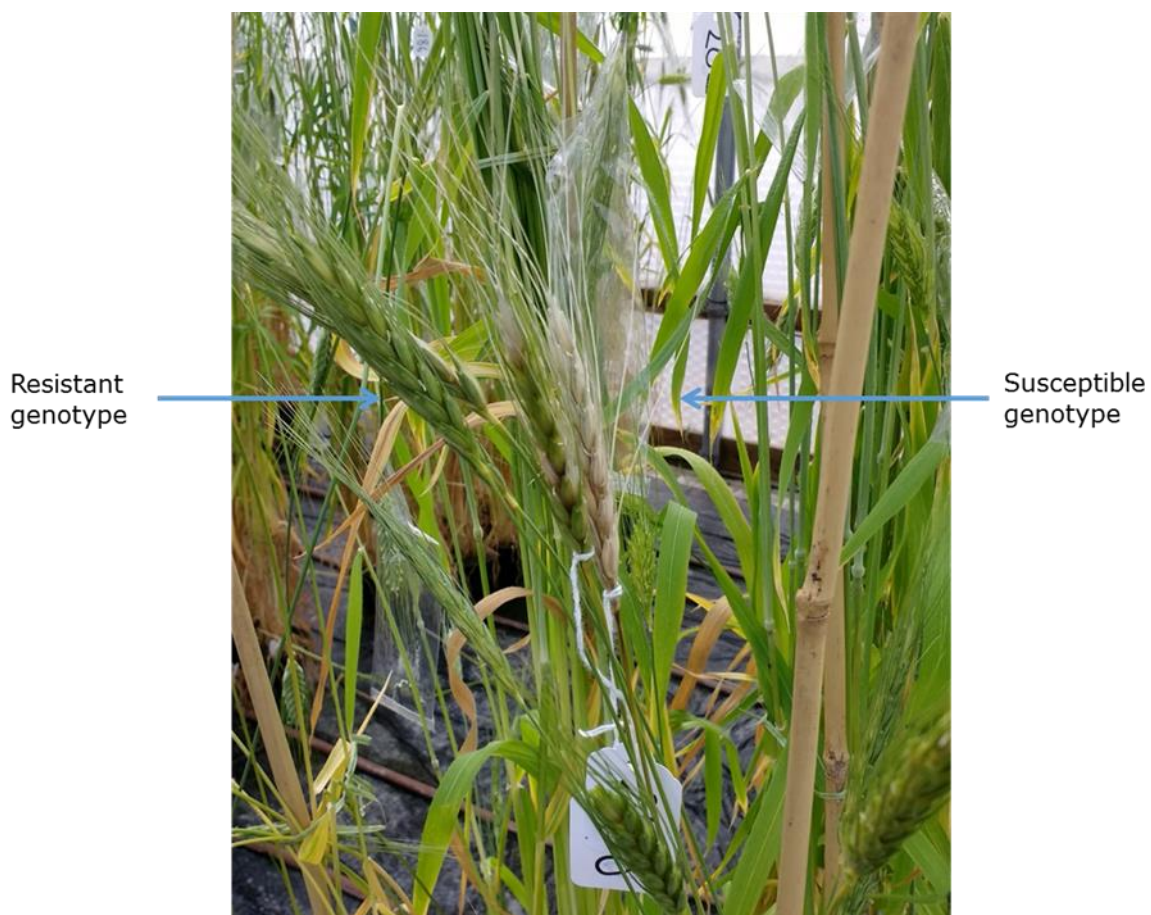
### **6.3.2 Poly tunnel trial 2017**

#### **1) Fusarium head blight resistance**

Disease was scored two times (16dpi, 19dpi) after spray inoculation to investigate response of the genotypes in two mapping populations to FHB across time. Disease severity of two mapping populations was recorded as the number of infected spikelets with disease symptoms. Disease data was obtained from 115 F<sub>2</sub> plants from population 1 and 223 F<sub>2</sub> plants of population 2. Overall infection scores for the moderately susceptible and highly susceptible genotypes

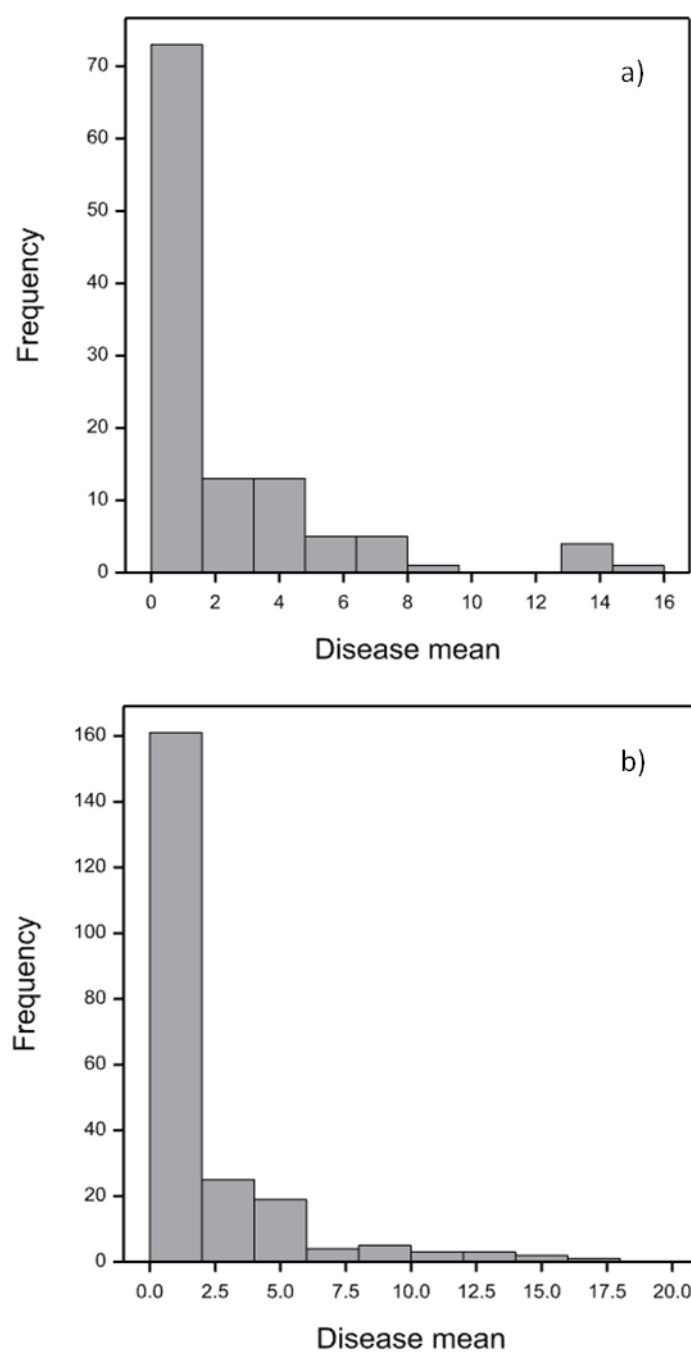
were generally higher at score 2 (19d) than at score 1 (16d) post inoculation, indicating noticeable disease progress during the second week after inoculation. The resistant genotypes however exhibited consistently extremely low levels of infection, with minimum disease development between 16 and 19d post inoculation. Mean disease scores of 19 dpi for each plant, were used for data analysis. Disease severity differed markedly among the genotypes in both populations (population 1 and population 2) with some plants exhibiting no disease symptoms while others were severely affected (Figure 6.8).

**Figure 6.8:** Heads of two lines 19 dpi with one 'resistant genotype' exhibiting no disease symptoms and the other 'susceptible genotype' severely affected by FHB.



The frequency of distribution of disease means for both populations are skewed towards resistance and allows differentiating between those plants with no symptoms at all and plants with any disease symptoms (of whatever severity). Frequency distributions of disease means for population 1 and 2 are shown in Figure 6.9.

**Figure 6.9:** Frequency distribution of Fusarium head blight disease mean collected under polytunnel conditions among a) 115 lines from the cross P95-99.1-1/538512 for population 1, b) 223 lines from the cross P95-99.1-1/355452 for population 2.



Disease severity ranged from 0 to 15.5 with a mean of 2.142 for population 1 (Figure 6.9), and was 0 for P95-99.1-1 and 6.684 for 538512. For population 2, disease severity ranged from 0 to 17.5 with a mean of 2.043 with same level of resistance for parent P95-99.1-1 and level of severity for 355452 as for population 1 parents, respectively. The populations had similar mean values and both were skewed towards resistance (Figure 6.9). There were some lines which were more susceptible than both parents 538512 and 355452, thus showing transgressive segregation in both populations. This indicates that the susceptible parent probably carries some resistance that is lacking in P95-99.1-1 so that some progeny have no resistance at all which manifests itself in disease scores above 6.68 in lines in the two populations.

For population 1, 44 lines showed complete resistance and 71 lines showed susceptibility for FHB. For population 2, 89 lines showed complete resistance and 134 lines showed susceptibility for FHB. The segregation ratio in both populations fitted a 9:7 distribution ( $\chi^2=1.403$ ,  $P=0.236$  for population 1 and  $\chi^2=1.348$ ,  $P=0.245$  for population 2, Table 6.3) indicating that the resistance of P95-99.1-1 is due to two independently segregating genes. The disease data also suggests that the susceptible parent in Population 1 (and probably also in Population 2) contains some sort of resistance as well but that this is not of type I (or it would probably not even be observed in the background of the huge P95-99.1-1 resistance effect) and is responsible for the highly type II susceptible out group.



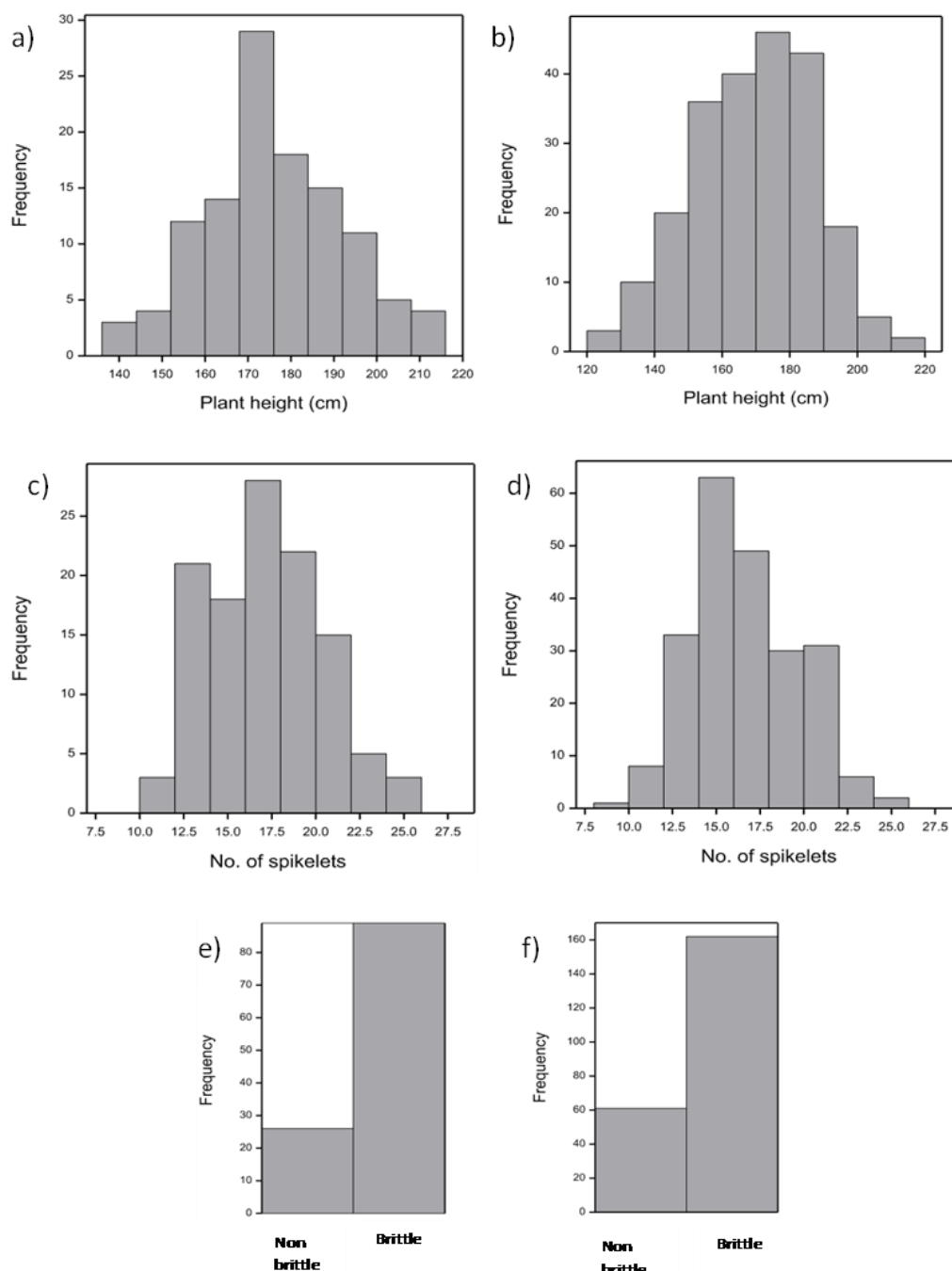
**Table 6.3:** Chi-squared analysis for disease means for 9:7 ratio for both mapping populations.

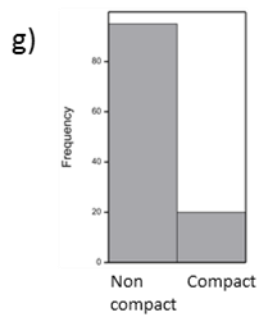
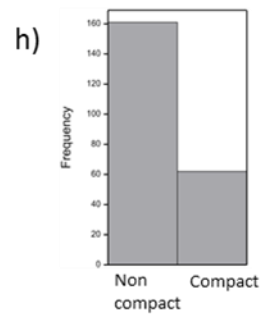
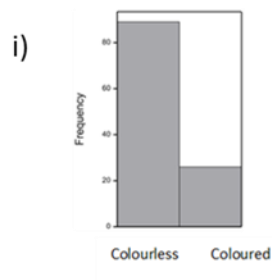
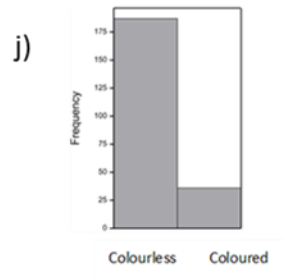
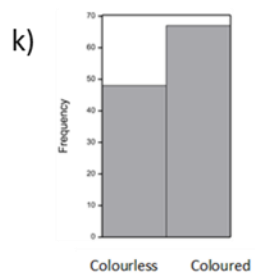
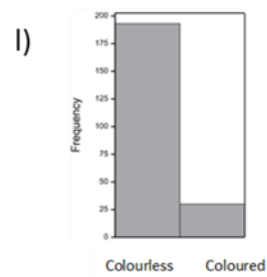
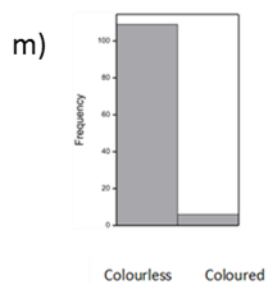
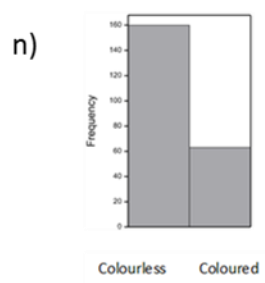
| Mapping populations | Category            | Observed | Expected | $\chi^2$ value | df | P value |
|---------------------|---------------------|----------|----------|----------------|----|---------|
| Population 1        | Resistant (score 0) | 44       | 50.3     | 1.403          | 1  | 0.236   |
|                     | Susceptible         | 71       | 64.7     |                |    |         |
|                     | Total               | 115      | 115      |                |    |         |
| Population 2        | Resistant (score 0) | 89       | 97.6     | 1.348          | 1  | 0.245   |
|                     | Susceptible         | 134      | 125.4    |                |    |         |
|                     | Total               | 223      | 223      |                |    |         |

## 2) Agronomic traits

High variation in plant height was observed among the genotypes under conditions in the polytunnel and the frequency distributions of both populations (population 1 and population 2) for plant height were continuous (Figure 6.10a and 6.10b). Plant height in the polytunnel ranged from 140 to 215 cm with an overall mean of 176.5 cm for population 1, and was 134.5 cm for P95-99.1-1 and 126 cm for 538512. Plant height for population 2 ranged from 120 to 220 cm with an overall mean of 170.3. High variation in number of spikelets was also observed among the genotypes and the frequency distributions of both populations for number of spikelets were continuous (Figure 6.10c and 6.10d). Number of spikelets for population 1 ranged from 11 to 26 with an overall mean of 17.7, and was 22 for P95-99.1-1 and 11 for 538512. For population 2, the number of spikelets ranged from 10 to 26 with an overall mean of 17.17.

**Figure 6.10:** Frequency distributions of agronomic traits among 115 lines of population 1 from the cross P95-99.1-1/538512 and 223 lines of population 2 from the cross P95-99.1-1/355452 under polytunnel conditions, plant height for a) population 1 and b) population 2, number of spikelets for c) population 1 and d) population 2, rachis (non-brittle/brittle) type) in population 1 and f) population 2, ear shape (non-compact/compact) g) in population 1 and h) population 2, awn colour (colourless/coloured) i) in population 1 and j) population 2, stem colour (colourless/coloured) k) in population 1 and l) population 2, head colour (colourless/coloured) m) in population 1 and n) population 2.





Frequency distributions and mode of inheritance of other agronomic traits, i.e. brittle/non-brittle rachis, ear shape (compact/non-compact) awn colour (colourless/coloured), stem colour (colourless/coloured) and head colour (colourless/coloured), were also investigated for both populations (Figure 6.10).

## 1) Rachis type

The parents of the F<sub>2</sub> mapping populations differed widely for rachis type with parent P95-99.1-1 having non-brittle rachis and 538512/355452 having brittle rachis. Thus, if a single gene with dominant effect was controlling the brittle rachis trait, in the F<sub>2</sub> population the AA, Aa and aA genotypes would all be brittle and the aa genotypes would all be non-brittle, leading to a 3:1 ratio. Out of 115 plants in population 1, 89 lines had brittle rachis and 26 lines had non-brittle rachis whereas for population 2, 162 lines had brittle rachis and 57 lines had non-brittle rachis out of 209 plants in total. The segregation ratio in both populations fitted a 3:1 distribution [ $\chi^2=0.349$ ,  $P=0.5$  for population 1 and  $\chi^2=0.122$ ,  $P=0.5$  for population 2, (see Table 6.4)] indicating that the brittle rachis trait is controlled by a single gene having a dominant gene effect.

**Table 6.4:** Combined Chi square tests for the different ratios tested for each trait.

| Mapping populations | Category                | Observed | Expected (3:1) | $\chi^2$ value | P value | Expected (13:3) | $\chi^2$ value | P value | Expected (15:1) | $\chi^2$ value | P value |
|---------------------|-------------------------|----------|----------------|----------------|---------|-----------------|----------------|---------|-----------------|----------------|---------|
| Population 1        | Brittle rachis          | 89       | 86.2           | 0.349          | 0.5     |                 | -              | -       |                 | -              | -       |
|                     | Non-brittle rachis      | 26       | 28.8           |                |         |                 |                |         |                 |                |         |
| Population 2        | Brittle rachis          | 162      | 164.2          | 0.122          | 0.5     |                 | -              | -       |                 | -              | -       |
|                     | Non-brittle rachis      | 57       | 54.8           |                |         |                 |                |         |                 |                |         |
| Population 1        | Ear shape (non-compact) | 95       | 86.2           | 3.587          | 0.058   |                 | -              | -       |                 | -              | -       |
|                     | Ear shape (compact)     | 20       | 28.8           |                |         |                 |                |         |                 |                |         |
| Population 2        | Ear shape (non-compact) | 161      | 167.2          | 0.919          | 0.337   |                 | -              | -       |                 | -              | -       |
|                     | Ear shape (compact)     | 62       | 55.8           |                |         |                 |                |         |                 |                |         |
| Population 1        | Coloured awns           | 26       | 28.8           | 0.351          | 0.55    |                 | -              | -       |                 | -              | -       |
|                     | Colourless awns         | 89       | 86.2           |                |         |                 |                |         |                 |                |         |
| Population 2        | Coloured awns           | 36       | 55.8           | 9.32           | 0.002   | 41.8            | 0.99           | 0.319   |                 | -              | -       |
|                     | Colourless awns         | 187      | 167.2          |                |         | 181.2           |                |         |                 |                |         |
| Population 1        | Coloured stem           | 67       | 86.2           | 17.18          | 0.0001  | 93.4            | 39.72          | 0.0001  | 107.8           | 246.6          | 0.0001  |
|                     | Colourless stem         | 48       | 28.8           |                |         | 21.6            |                |         | 7.2             |                |         |
| Population 2        | Coloured stem           | 30       | 55.8           | 15.85          | 0.0001  | 41.8            | 4.1            | 0.0429  |                 | -              | -       |
|                     | Colourless stem         | 193      | 167.2          |                |         | 181.2           |                |         |                 |                |         |
| Population 1        | Coloured head           | 6        | 28.8           | 24.003         | 0.0001  |                 | -              | -       | 7.2             | 0.213          | 0.644   |
|                     | Colourless head         | 109      | 86.2           |                |         |                 |                |         | 107.8           |                |         |
| Population 2        | Coloured head           | 63       | 55.8           | 1.257          | 0.262   |                 | -              | -       |                 | -              | -       |
|                     | Colourless head         | 160      | 167.2          |                |         |                 |                |         |                 |                |         |

## **2) Ear shape**

Parent P95-99.1-1 is characterized by a compact, dense and flat ear shape whereas both parents 538512 and 355452 have non-compact ear shape. Both the populations individually were tested for 3:1 ratio for two types, namely compact and non-compact. The segregation ratio for both populations did fit a 3:1 ratio ( $\chi^2=3.587$ ,  $P=0.058$  for population 1,  $\chi^2=0.919$ ,  $P=0.337$  for population 2, Table 6.4) indicating that the ear compactness trait is controlled by a single gene having a dominant gene effect.

## **3) Awn colour**

With respect to awn colour 89 lines were colourless and 26 lines were coloured (brown/black) for population 1. Thus, if a single gene with dominant effect was responsible for producing the colourless awns, the AA, Aa and aA genotypes would produce colourless awns phenotype and aa genotype would produce coloured (brown/black) awns phenotype, leading to a 3:1 ratio. The segregation ratio in this population did fit a 3:1 ratio ( $\chi^2=0.351$ ,  $P=0.55$ , Table 6.4) which suggests that awn colour is controlled by single dominant gene effect. For population 2, 187 lines were colourless and 36 lines were coloured (brown/black). The segregation ratio in this population did not fit a 3:1 ratio ( $\chi^2=9.32$ ,  $P=0.002$ ) which suggests that in population 2 the nature of the genetic control of awn colour is complex. The segregation ratio did, however, fit a 13:1 ratio for this population ( $\chi^2=0.99$ ,  $P=0.319$ , Table 6.4) which is compatible with a dominant epistasis effect. If the expression of the gene producing the colourless phenotype and the suppressor of this gene are both dominant traits, the genotypes where both the dominant gene and suppressor genes or either of them are present will produce colourless phenotype whereas

the genotypes where the recessive allele of both genes are present will produce coloured (brown/black) phenotypes, leading to 13:1 ratio.

#### **4) Stem colour**

The two populations were observed to differ in their genetic basis of control for differences in the two colour traits i.e. awn colour and stem colour. Most of the lines (18 lines out of 26) which showed the brown/black coloured awns also had red/purple coloured stems in population 1. For stem colour, 67 lines showed red/purple coloured stem and 48 lines were colourless for population 1. Thus, if a single gene with dominant effect as noted for awn colour was controlling the stem colour, 3:1 ratio is hypothesized. The segregation ratio in this population did not fit a 3:1 ratio ( $\chi^2=17.18$ ,  $P=0.0001$ , Table 6.4). It was further tested for 13:3 and 15:1 ratios as well but did not fit either of the two (Table 6.4). The inability to identify a segregation ratio to explain the genetic basis of the stem coloration may reflect difficulties in accurately determining stem colour in many lines.

For population 2, 193 lines were colourless and 30 lines showed coloured (red/purple) stem. The segregation ratio in this population did not fit a 3:1 ratio ( $\chi^2=15.85$ ,  $P=0.0001$ ) which indicates the genetics underlying this trait is complex. If the expression of the dominant gene producing the colourless phenotype is suppressed by another dominant gene, as found for awn colour, it is expected to segregate in 13:3 ratio. Thus the ratio of 13:3 was tested for this population. The segregation ratio almost fits a 13:3 ratio ( $\chi^2=4.1$ ,  $P=0.0429$ , Table 6.4) expected for a dominant epistasis effect.

## 5) Head colour

Population 1, parent P95-99.1-1 with colourless heads was crossed to parent 538512 with coloured head. The observed value among the  $F_2$  for population 1 was, 6 lines showed brown/black coloured heads whereas 109 lines showed colourless head and for population 2, 63 lines showed brown/black coloured heads and 160 lines were colourless. The ratios differ for the two populations which suggest that the genetics controlling this trait is different for the two populations. Chi square test in population 1 for 3:1 ratio did not support the hypothesis of single dominant gene controlling this trait. Further it did not fit 13:3 ratio so, the ratio of 15:1 was tested for population 1. The segregation ratio did fit a ratio of 15:1 ( $\chi^2=0.213$ ,  $P=0.6441$ , Table 6.4) which leads to the acceptance of null hypothesis of duplicate gene action, that there are two interacting genes responsible for controlling this trait. For population 2, the segregation ratio fitted a 3:1 ratio ( $\chi^2=1.257$ ,  $P=0.262$ , Table 6.4) which leads to the acceptance of the null hypothesis that single gene with dominant effect is responsible for the difference in head colour in the parents of population 2.

### Correlations between colour traits

The correlations among awn colour, stem colour and head colour for both populations were also noted (Table 6.5).

**Table 6.5:** Pearson correlation coefficients between awn colour, stem colour and head colour among 338 lines from the cross P95-99.1-1/538512, population 1 and P95-99.1-1/355452, population 2 using the polytunnel data.

| Traits                      | Population 1 | Population 2 |
|-----------------------------|--------------|--------------|
| <b>Awns and stem colour</b> | 0.0391*      | 0.0306*      |
| <b>Awns and head</b>        | 0.4007       | 0.0141*      |
| <b>Stem and head</b>        | 0.3941       | 0.1950       |



Awn colour and stem colour showed correlation for both populations (Table 6.5). Analysis of data sets for awn colour and head colour for both populations was carried out. There was no correlation observed between awn colour and head colour for population 1 but the two traits appeared correlated in population 2 (Table 6.5). Analysis of data sets for stem colour and head colour from both populations showed no significant differences and there was no relationship observed between stem colour and head colour (Table 6.5).

### 3) Association of agronomic traits with FHB

The correlations among FHB resistance and various agronomic traits were also observed for both populations. For both populations, FHB was highly significantly associated with ear shape and to a lesser extent with plant height and rachis type. FHB score was also highly associated with the number of spikelets in the head for population 1 ( $P=0.0079$ ) but not in population 2. FHB severity was not associated with awn, stem or head colour in either population (Table 6.6).

**Table 6.6:** Pearson correlation coefficients between agronomic traits and Fusarium head blight means among 338 lines from population 1 (P95-99.1-1/538512) and population 2 (P95-99.1-1/355452), using the polytunnel data.

| Agronomic traits           | Disease mean |              |
|----------------------------|--------------|--------------|
|                            | Population 1 | Population 2 |
| <b>Rachis type</b>         | 0.0004***    | 0.0145*      |
| <b>Ear shape</b>           | 0.0096**     | <0.0001****  |
| <b>Plant height</b>        | 0.0130*      | <0.0001****  |
| <b>Number of spikelets</b> | 0.0079**     | 0.4280       |
| <b>Awns colour</b>         | 0.1217       | 0.8263       |
| <b>Stem colour</b>         | 0.1557       | 0.8944       |
| <b>Head colour</b>         | 0.9903       | 0.0590       |

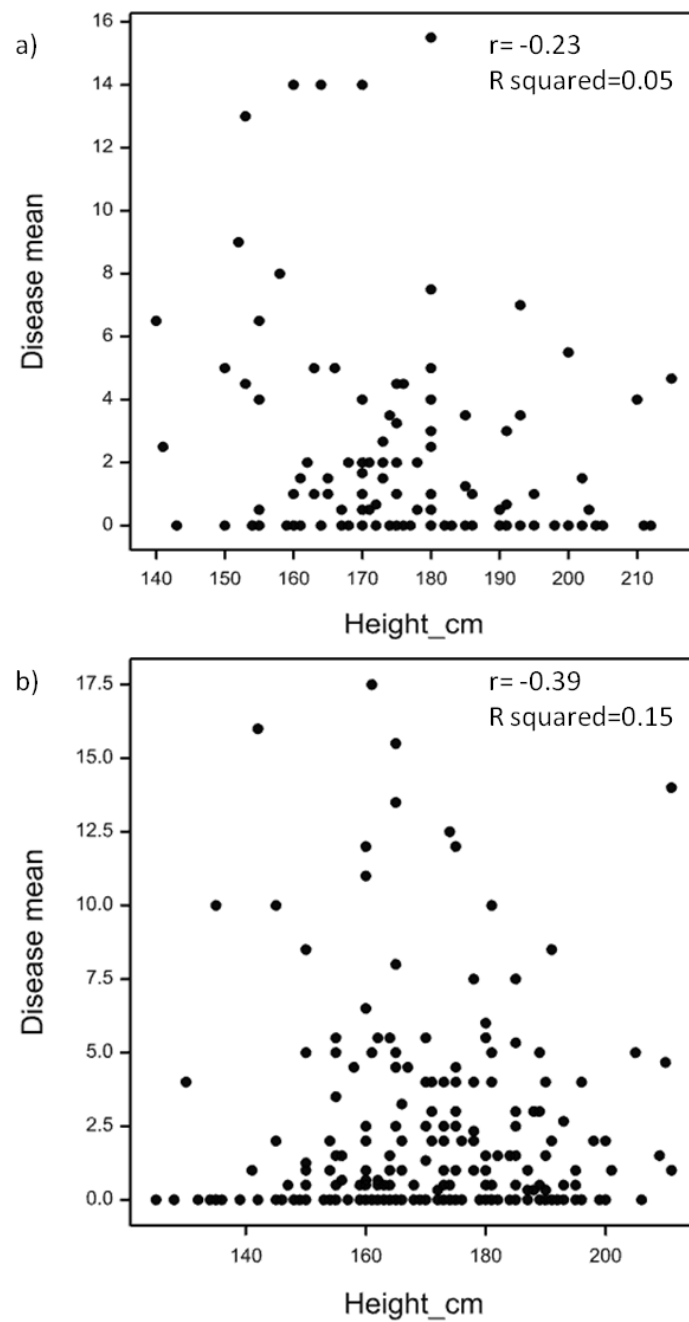
Analysis of data sets from both population 1 and population 2 showed significant differences between brittle and non-brittle genotypes for disease means. Lines that were non-brittle like the P95-99.1-1 parent had significantly less FHB

disease i.e. the genotypes with the non-brittle rachis were more resistant to FHB. Here, the R squared value appeared very low ( $r^2=0.104$  for population 1 and  $r^2=0.027$  for population 2) which suggests weak association between the two traits.

Analysis of data sets from population 1 showed that FHB was highly significantly associated with the non-compact and compact ear shapes in both populations (Table 6.6). Lines with compact, dense ear shapes like the P95-99.1-1 parent had significantly less FHB disease. However, the R squared value seem to be very low ( $r^2=0.057$  for population 1 and  $r^2=0.079$  for population 2), which indicates that there is a weak association between the two traits (with less variation) in both the populations.

Plant height was negatively correlated with resistance to FHB in both populations with lines that were taller being more resistant (Table 6.6 and Figure 6.11). Although the two traits are correlated it should be noted that the R squared values are incredibly low ( $r^2=0.05$  for population 1 and  $r^2=0.15$  for population 2) so the effect of height only explains a very small amount of the variation in resistance.

**Figure 6.11:** Scatter plot of disease means versus plant height for a) population 1 and b) population 2, respectively.



Number of spikelets on a head of each line was also studied for both populations in the polytunnel. Analysis of data sets from population 1 showed that FHB was highly significantly associated with the number of spikelets in population 1 but not in population 2 (Table 6.6).

Analyses of data sets for all the three colouration traits (awn colour, stem colour and head colour) from both populations showed no significant association with FHB score in either population (Table 6.6). Hence, there was no relationship observed between awn colour, stem colour, head colour and FHB.

## 6.4 Discussion

For the past two decades, wheat production has been threatened by FHB and has caused serious yield and end-use quality losses. Breeding for FHB resistance is a high priority of wheat breeding programs globally as only few sources of useful resistance are available. Most resistances reported for FHB are primarily of type II (Anderson *et al.*, 2001) and research has generally focused on this type of resistance as it has been considered both simple to target and more robust than type I (Buerstmayr *et al.*, 2003). In the present study, *T. timopheevii* subsp. *timopheevii*, accession P95-99.1-1 expressed significant type I FHB resistance under polytunnel conditions. Therefore, P95-99.1-1 is a promising source of FHB resistance for wheat breeders targeting this important trait as it can provide considerable protection under intense disease pressure from the initial infection by the fungus.

Under natural conditions, FHB occurs unpredictably and the disease is not uniformly spread across the field (Buerstmayr *et al.*, 2002). Therefore, artificial inoculation is essential for a reliable FHB resistance evaluation and to detect QTLs in a mapping population. Spray inoculation of individual heads at mid-anthesis was done in the polytunnel for 2017 trial to provide an overall measure of resistance to both initial infection and the spread of the disease within a spike. Thus, spray inoculation is a simple and reliable method to evaluate type I + type II resistance (Yang *et al.*, 2005).

Disease severity differed markedly among the genotypes in both populations as almost half of the plants were completely resistant with the other half being either moderately susceptible or highly susceptible to FHB. This suggests that the resistance of P95-99.1-1 is relatively simply inherited and may be controlled by two independently segregating genes in *T. timopheevii* accession P95-99.1-1.

The resistance shown in the present study is of novel type I, where the fact that the lower ratio (i.e. the 7 out of the 9:7 ratio) forms the 'resistant' group makes it look as though the resistance may be controlled by recessive gene effects where loss of the allele for susceptibility gives resistance. Susceptibility factors to FHB in wheat has been reported previously by Garvin *et al.*, (2015) where wheat cultivar Apogee was reported to have FHB susceptibility genes that promoted disease spread in infected spikes, and their elimination increased FHB resistance in a novel manner. Chen *et al.* (2009) reported that ethylene signalling in wheat during infection by fungus plays a role in FHB susceptibility, and interfering with ethylene signalling increases resistance.

Malihipour *et al.* (2017) mapped a major QTL for type II FHB resistance on chromosome 5AL which originated from *T. timopheevii*, in a RIL population between AC Brio and TC 67. Further, QTL analysis and mapping is needed to confirm if the QTL or gene for type II resistance that appears to be present in the susceptible parents is the same or lies in the same chromosome region as reported by Malihipour *et al.*, (2017) or is a novel gene different from it.

In the present study, plant height showed negative relationship with disease means in both populations following spray-inoculation of single-florets in polytunnel experiment. Taller lines tend to be more resistant but the effect was extremely small with respect to overall differences in resistance. This contrasts to the situation in wheat where the negative association between height and FHB susceptibility generally highly significant in field trials (Mesterhazy, 1995; Gervais *et al.*, 2003; Somers *et al.*, 2003; Paillard *et al.*, 2004; Steiner *et al.*, 2004; Wilde *et al.*, 2007; Srinivasachary *et al.*, 2008; Haberle *et al.*, 2009; Malihipour *et al.*, 2016, 2017).

Non-brittle rachis is thought to have been the first symptom of wheat domestication (Peleg *et al.*, 2011). Spikelet shattering in wheat has been reported to be a dominant character (Love and Craig, 1919; Peleg *et al.*, 2011). In the present study, chi squared analyses for both populations for rachis type fitted a 3:1 ratio which suggests that brittle rachis (*Br*) trait is controlled by a single dominant gene. In the past, numerous analyses of rachis brittleness in hexaploid and tetraploid wheat has shown that *Br* is controlled by loci on homoeologous group 3 (Watanabe *et al.*, 2006). Further, Rawat *et al.* (2012) mapped the gene *Br-At1* in the recombinant inbred lines population of *T. araraticum*-*T. timopheevii* on chromosome arm 3A<sup>t</sup>S. Thus, it is expected that the dominant gene for rachis type is present on chromosome arm 3A<sup>t</sup> or homoeologous group 3 chromosomes in parents of both population under study. The strong association of rachis type with disease severity in population 1 suggests that the resistance to FHB may be linked to this gene (either 3A<sup>t</sup> or 3G).

Ear compactness in wheat is governed by several major genes as well as QTL, where the gross ear morphology is under the control of three major genes, *Q*, *C* and *S1* located on chromosomes 5A, 2D and 3D respectively, but also of minor genes (Ausemus *et al.*, 1967; Sourdille *et al.*, 2000). Genotypes in two populations showing type 4 were classified in 'compact' class as shown for parent P95-99.1-1 and genotypes showing type 1, 2 and 3 were classified in 'non-compact' class as shown by parents 538512 and 355452. Chi squared analysis did fit a 3:1 ratio for both populations suggesting that compactness (*C*) is controlled by a single dominant gene. The observed differences in laxness such as type 2 and 3 are probably controlled by genes of minor effect. The strong association of ear shape and disease severity in both populations

suggests that the resistance is linked to gene(s) responsible for ear compactness. Strong association between QTL for resistance with ear compactness was also found by Schmolke *et al.*, (2005) where he detected four QTLs for resistance to disease severity and one out of these QTLs was associated with ear compactness. In that instance increased FHB resistance was associated with laxness whereas in the current study increased FHB resistance was associated with compactness.

Furthermore, analyses for awn colour in population 1 showed that the trait is controlled by single dominant gene. Previous studies demonstrates that genes controlling both glume (represented as head colour in present study) and awn colour in wheat are located on the short arms of the homoeologous group 1 chromosomes, designated as *Rg1* (1BS), *Rg2* (1DS) and *Rg3* (1AS) (McIntosh *et al.*, 2003). In addition, according to Panin and Netsvetaev, (1986) black awns is determined by the three complementary genes *Bla1*, *Bla2* and *Bla3* which were mapped to the distal region of chromosomes 1AS, 1BS and 1DS, respectively (Borner *et al.*, 2002). Thus, it is expected that the dominant gene for awn colour is present on either chromosome arm 1A<sup>t</sup> or 1G in parents of population 1 under this study.

Genes controlling glume colour (represented as head colour in present study) in wheat are also located on the short arms of the homoeologous group 1 chromosomes, designated as *Rg1* (1BS), *Rg2* (1DS) and *Rg3* (1AS) (McIntosh *et al.*, 2003). Another gene *Brg* (brown glume) was mapped to distal end of 1DS (Koval, 1994) which was later found to be allelic to *Rg2*. *Bg* (black glume) gene was further located to chromosome 1A and was supposed to be allelic to *Rg3* (Borner *et al.*, 2005). Awn colour is thought to be genetically associated with



glume colour, which is in agreement with our results as both traits showed correlation for population 2 although this was not evident in population 1.

There was no relationship between head colour and stem colour which indicates that the head colour is controlled separately by different genes and has a different genetic control.

Pigmentation of different plant organs is important for plant adaptation to some stressors. Purple pigments protect plant tissues from UV irradiation and are implicated in tolerance to drought, heavy metals, and pathogens (Gould, 2004). In wheat, purple pigments, caused by the anthocyanins are found in the culm (stem) (gene designation *Pc*), leaf blade (*Plb*), auricle (*Ra*), leaf sheath (*Pls*), pericarp (*Pp*), coleoptile (*Rc*) or anther (*Pan*) (McIntosh *et al.*, 2008). The genes *Rc*, *Pc*, *Pan*, *Plb* and *Pls* are closely linked to each other and represent homoeoloci on the short arms of chromosome 7A, 7B, and 7D (Blanco *et al.*, 1998; Maystrenko, 1992; Khlestkina *et al.*, 2002a, b, 2008, 2009). This supports the present study, as chi squared analyses for both populations for stem colour did not fit a 3:1 ratio which suggests that the trait is controlled by complex genetics.

Overall, the evidence indicates that the two populations have similar genetic basis for the differences in brittle rachis but that they differ in the genetic basis of the differences between the parents for many of the other traits.

Detailed phenotypic analysis of disease and various agronomic traits did help to understand the basic genetics but is not at this stage very suitable for exploitation, hence mapping of the genes with the help of KASP markers is underway. Once the maps are available various QTL responsible for disease resistance and other traits can be analysed and detected. QTL mapping will aid in locating the position of the genes responsible which will hence provide

markers for breeders to use and researchers to ultimately clone the genes responsible. The QTL for type I resistance will thus provide an alternative for the currently known QTLs or may be combined with them to enhance the level of resistance to FHB in wheat cultivars. *T. timopheevii* is considered to be a source of resistance for several important diseases of wheat e.g. rust resistance apart from FHB. Therefore, transfer and use of *T. timopheevii* QTL for FHB in wheat germplasm may enhance resistance to other diseases as well. However, the negative correlation between FHB and undesirable agronomic traits such as plant height may impede breeding wheat for resistance to FHB using P95-99.1-1. Lines containing whole chromosome additions, substitutions and introgressed segments have been developed with *T. timopheevii* using the whole genome transfer approach. Lines are still segregating and selfing is underway to make them homozygous. Once the lines are made homozygous, they will then be screened for FHB resistance targeting the lines having the chromosomes responsible for FHB resistance as identified in the QTL analyses of the populations.

## **Chapter 7**

### **General discussion and conclusion**

The development of wheat genotypes for future crop improvement will require the selection of wheat cultivars that meet new challenges of global warming and increasing population. Introducing new genetic variation is one pathway to the development of new cultivars. To introduce new genetic variation via introgression, wheat breeding programmes will need to screen the wild relatives of wheat with the aim to ultimately introgress this new germplasm into the elite wheat cultivars (Wulff and Moscou, 2014).

The primary goal in exploiting the genetic variation of wild relatives is to introduce new genetic variation by introgression from the wild relative genetic resources into adapted germplasm with new breeding tools/methods. These include new tools of genotyping along with phenotyping which will enable plant breeders to screen for genes important for breeding programmes and hence characterise genetic resources at the gene level. This will provide insights into the allelic content of the germplasm, potentially useful for breeding.

The work outlined in this thesis provides only a starting point for the possibilities that are available with the *T. timopheevii*/*T. aestivum*. The work has shown that recombination has occurred between the wheat and *T. timopheevii* chromosomes, leading to introgressions. Genetic maps of the A<sup>t</sup> and G genome have been produced and bins consisting of large number of markers are distributed along the whole length of the chromosome, although, there are still gaps present. Due to the large genome complexity and sequence redundancy, high-quality reference genome assemblies were not available for wheat, when the SNPs used in the programme were designed to characterize introgressions.

Exome capture was performed on the cDNA of ten wild relatives and four wheat varieties where the DNA sequences from the conserved genes from each of the wild relative and wheat were then compared to produce single nucleotide polymorphisms i.e. SNPs. Due to this, only a partial representation of the genomes were captured. A significant number of expressed genes specific to *T. timopheevii* may be absent, leading to less SNPs which are *T. timopheevii* specific and giving rise to gaps in the genetic maps. Thus, there is an urgent need to understand the genome organization in *T. timopheevii* and increase the number of genome-specific molecular tools and identify loci underlying traits of interest.

An extension of this work will be high-resolution genetic and physical maps of *T. timopheevii*/*T. aestivum* chromosomes and the establishment of synteny for each of the individual chromosomes. High throughput of the next generation sequencing technology now available makes it possible to sequence the genomes, where the problems of assembling the large genome of wheat with long reads have been resolved. An improved assembly and annotation of the wheat genome, representing >78% of the genome recently became available (Clavijo *et al.*, 2017) which identified complete families of agronomic genes in wheat. Once the *T. timopheevii* is sequenced, using this assembly the sequence of *T. timopheevii* can be blast to wheat. Targeting only those genes between *T. timopheevii* and wheat, anything that does not give hit in wheat or not present in wheat will be *T. timopheevii* specific. Therefore, targeting only genes which are *T. timopheevii* specific, large number of SNPs can be designed which will thus enhance the genome coverage of *T. timopheevii* maps.

The decreasing costs of sequencing can finally allow us to sequence the accession of *T. timopheevii* used in this programme for producing introgressions,

followed by sequencing of several different accessions to look for *T. timopheevii* specific genes which would give a better genetic coverage. This can be achieved using the whole genome shotgun strategy using high-throughput genotyping platforms which are now available and has been used to sequence several genomes. Draft genome sequences of *T. urartu*; the A genome donor of wheat (Ling *et al.*, 2013) and *Ae. tauschii*, the D genome donor of wheat (Luo *et al.*, 2013) were made available using this approach. However, when sequencing of *T. timopheevii* has been achieved it will be possible to identify genes of known function in wheat. Orthologous genes will most likely have similar regulation, genetic mechanisms and biochemical pathway homologues such that trait manipulation strategies may be extrapolated to the *T. timopheevii* chromosomes.

An alternative approach for genotyping could be the use of flow cytometry, where the genome can be dissected into individual chromosomes by flow cytometric sorting (Dolezel *et al.*, 2007). Flow sorted chromosomes which are suitable for NGS (Next generation sequencing) to establish linear gene order and assess gene synteny with other species has been demonstrated in wheat, barley and rye (Mayer *et al.*, 2011; Martis *et al.*, 2013; IWGSC 2014). Giorgi *et al.* (2013) advanced this technique for use in introgression programmes with FISHIS (FISH in suspension), which fluorescently labels specific microsatellite sequences on chromosomes in suspension. Fluorescent labelling of chromosomes with microsatellite repeats, as probes for FISH is done in suspension prior to flow cytometric analysis to facilitate sorting all chromosomes from the wild relative genome. DNA amplified from flow sorted chromosomes is then used for PCR with molecular markers to obtain an insight into the macrosyntenic relationships between the genome of wild relative and bread wheat at the chromosome level.

This technique has recently been successfully exploited by Molnar *et al.*, (2016) to dissect the U, M, S and C genomes of wild relatives of Aegilops species (*Ae. umbellulata*, *Ae. comosa*, *Ae. speltoides* and *Ae. markgrafii*). Application of this technique to the current work could be in different ways: The FISH Karyotype of *T. timopheevii* was developed by Miko *et al.*, (2015) which held true for the accession used in the current study, using all the probes used to identify chromosome of *T. timopheevii* in past in addition to the one used in the current study, FISHIS can be applied to sort all the chromosomes from the two genomes of *T. timopheevii*. Characterization of individual chromosomes of *T. timopheevii* by flow sorting will thus help in confirming the addition and translocation lines of *T. timopheevii*. Further, SNP markers specific to *T. timopheevii* chromosomes can be used to study the syntenic relationship between the genomes of *T. timopheevii* and wheat.

The primary justification in breeding for the development of genetic maps is the phenotype mapping. DNA markers closely linked to a gene responsible for a desirable phenotype are an important tool for indirect selection in breeding programmes. Such indirect screening methods have several advantages for the plant breeders. Screening can be carried out at a very early developmental stage and therefore reduces the demands on glasshouse space, field trials etc. In many cases the screening for a molecular marker may be considerably easier than the screening for a particular phenotype. This would be particularly true for those phenotypes apparent only under certain environmental conditions. Linkage of molecular markers can also be used to look for particular combinations of alleles including combinations occurring together at very low frequencies.

The use of molecular markers for indirect selection in breeding programmes requires the marker system used to meet certain criteria. Markers need to be

fairly evenly distributed throughout the genome covering each chromosome from end to end. The markers must also be at a sufficient density to ensure reasonably close linkage to the trait or traits of interest. The analysis of SNP distribution presented in this thesis suggests that a considerable amount of care needs to be taken in establishing marker distribution before the marker system is utilized for breeding purpose. Screening will be difficult for breeders. The different problems which will be faced by breeders include:

1) Functional aspect of wild relative gene transfer: There is a complex relationship between the wild relatives and host genes (Wulff and Moscou, 2014) and, as a result, in some cases the wild relative genes may not function in the host genome as expected or will get suppressed. Riley and Macer. (1966) found that resistance to wheat stem rust from rye functioned poorly in wheat and resistance to wheat leaf rust did not function at all. It is more likely that the polyploid nature of wheat impact wild relative gene expression. Khalil *et al.*, (2015) studies showed that genes with low similarity between rye sequences and their closest matches in the Triticum genome have a higher probability to be repressed or deleted in the allopolyploid genome. Hence, the identification of genes and the analysis of their distribution would also be of interest in establishing the presence or absence of any relationship between gene type and genome location before the breeders can use them.

2) The material produced in the current work needs to be handled with the pre-breeders who have expertise in handling the material which is not developed specific to one target trait. The main aim of most breeding companies is yield, which is not targeted in the current programme so the pre-breeders need to look at the material produced in the current study with more open eyes rather than just yield.

3) Backcross breeding is a very long process, often many generations of backcrossing are required to decrease linkage drag and other wild characteristics. Years are often required for release and acceptance of varieties.

4) Molecular markers tightly linked to the target traits are much needed, which will save both time and money for the breeders to exploit the wild relative introgression material.

A major drawback of the *T. timopheevii*/wheat introgression material produced in the programme is that many of these introgression lines carry large introgressed chromosome segments. These large introgressions that carry a target gene will often also carry agronomically deleterious genes that make the line of little or no agronomic/commercial importance. The solution to the above problem is to reduce the size of the introgressed segment so that the deleterious genes are removed and the target gene(s) is retained. Two lines with large overlapping introgressions where the target gene was located in the overlap could be intercrossed (Sears, 1972). During gametogenesis in the resulting hybrid, recombination would occur between the two introgressions such that the individuals recovered in the progeny would carry a greatly reduced small introgression on which the target gene was located. This method could lead to the generation of large numbers of plants with small introgressions with target genes, screening with the relevant markers (chromosome specific KASP markers) would be a relatively time efficient procedure, enabling targeting of those regions of the chromosome that carry genes for useful agronomic traits for breeders to exploit.



## **Other ways *T. timopheevii*/*T. aestivum* can be exploited/alternatives to breeding programmes**

### **1) Double haploid production**

Introgression lines carrying large chromosome segments produced in the present work can be subjected to production of double haploids (DH). This method is used to produce homozygous progeny from the backcross generated lines in a single step. This technology would be a promising tool not only for cultivar development, but also for pre-breeding and for the establishment of mapping populations (Laurie and Bennett, 1988). DH is widely used in wheat, barley, rice and maize (Dunwell, 2010; Dwivedi *et al.*, 2015; Forster *et al.*, 2007; Germana, 2011).

### **2) Integration of Cisgenesis with CRISPR** (clustered regularly interspaced short palindromic repeat)

Cisgenesis is a transformation technique in which DNA fragments from wheat or cross-compatible species are incorporated into the genome. As wheat has many wild and cultivated relatives, the prospects for developing precision breeding via the cisgenesis technology are promising. This technology would be a modern alternative to traditional breeding and would offer an ideal way of incorporating agronomically important genes from wild relatives into wheat. This technique can overcome problems such as linkage drag and crossing barriers. A classic example is the successful use of cisgenesis to confer resistance to late blight in potatoes (Haverkort *et al.*, 2009). This method has also been used to transfer a gene coding for a MYB transcription factor isolated from *Th. intermedium* and conferring to the transformed wheat enhanced resistance to take-all disease (Liu *et al.*, 2013). In the present work, once desirable variant alleles from *T. timopheevii* are identified with the help of the introgression lines produced, they

could, possibly, be made available for cisgenesis manipulations (although these lines will retain their inherent drawback of random insertion and unpredictable expression of the desired gene in the recipient genome) Or they could serve as externally supplied DNA templates for targeted gene/allele correction or replacement via CRISPR/Cas 9 (CRISPR associated 9) a genome editing approach (Podevin *et al.*, 2013).

Besides mapping applications, *T. timopheevii*/*T. aestivum* introgression lines may be used in other areas of research. Studies on the mineral concentrations, in *T. timopheevii* accessions, have shown the potential of *T. timopheevii* to be exploited for breeding wheat for micronutrients. Only one replication for assessing micronutrient concentrations in introgression lines was feasible due to their segregating nature, i.e. heterozygous introgressions. Future experiments with more replicates can be done once the lines are made homozygous to validate current results. This will enable specific genes responsible for higher micronutrient concentrations to be identified. QTL mapping will then be needed to confirm the results. The availability of high-density genetic and physical maps will allow this trait to be located to a specific chromosome region or regions and opens up the possibility of further using this trait for breeding purposes.

Micronutrient analysis in the present work focused on only five minerals out of the 20 minerals analysed. Future studies can concentrate on looking at the micronutrient concentrations of other minerals such as silicon and potassium, to exploit the potential of *T. timopheevii* to the fullest. *T. timopheevii* was also found to be resistant to FHB in present study. QTL mapping studies will reveal if there exist a QTL(s) present on the same genomic region which is responsible for resistance to FHB and high micronutrient content. If the QTLs map to

different regions or chromosomes, these two can be pyramided, and transferred to wheat, enhancing wheat breeding programmes.

Studies on assessing *T. timopheevii* accessions for Fusarium head blight resistance were also carried out. The results show the presence of a type I resistance present in *T. timopheevii*. This form of resistance is difficult to find, thus the resistance found is novel. QTL mapping is required to confirm the present results. The segregating data from the F<sub>2</sub> population is very interesting indeed as the 7:9 ratio indicates the involvement of two genes responsible for resistance (both acting as recessive). Hence, combining the high type I resistance from *T. timopheevii* with something like *Fhb1* type II resistance could be a way to get very highly FHB resistant wheat.

*T. timopheevii* has shown to have many desirable traits with the present study demonstrating the huge genetic variation present for mineral concentrations and also FHB resistance. Future studies on other useful target traits such as quality traits etc. would further provide an extremely important resource for both breeding purposes and future improved wheat varieties. Identification of genes controlling the useful agronomic traits and quality traits using *T. timopheevii* introgression lines will enhance breeding of wheat in the future. A consequence of developing the *T. timopheevii*/*T. aestivum* recombinant chromosome lines is that many lines of *T. aestivum* will be produced with a single, small *T. timopheevii* chromosome segment carrying an important gene(s).

### **Lessons learned**

In the present work, a shot gun approach was followed at the start of the programme which has both positive and negatives compared to other work done in the wild relative introgression field. In the past, introgression work has largely focused on screening for one single target trait from a particular wild relative

ignoring any other useful traits which might have been present. The merit of this approach was it enhanced the wheat breeding for particular traits but it came with a drawback of wasting huge resources which might have been useful for other traits as well. The shot gun approach followed in this programme was the first attempt to capture the full potential of *T. timopheevii* without targeting a particular trait with all the tools available at that time. Advances in technology and the decrease in the cost of sequencing since the start of this programme might have resulted in a different approach being taken. The wild relatives could have been screened phenotypically before using them for the introgression programme or sequenced first to reduce the time of exploitation of this material as novel genes from wild relatives could have been targeted for breeding programmes straightway.

One of the potential problems with a programme such as the one described in this thesis is the understanding required of a wide range of techniques from chromosome manipulation via the crossing programmes to the lab based molecular work on markers and GISH. This has resulted in a limited number of scientists working in this field, not just in the UK but worldwide despite the major advantages such work could have for future wheat breeding.

### **Concluding remarks/Summary**

- 1) As a result of this programme, large numbers of *T. timopheevii*/*T. aestivum* introgression lines have been developed.
- 2) A total of 1,807 introgressions were generated between *T. timopheevii* and wheat; 942 from the A<sup>t</sup> genome and 865 from the G genome.
- 3) Development of KASP SNP markers is underway which will aid in the further characterization of the introgressions produced.

4) The potential of the work on *T. timopheevii* for both mineral concentrations and disease resistance demonstrates that the introgression lines could be a very valuable resource for future wheat breeding programmes.

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## **Appendix 1**

### **Buffers and Reagents**

#### **5% C<sub>l</sub>NaO** (sodium hypochlorite)

Stock                                      10% solution

Use (5% for 50ml)              25ml stock + 25ml water + 1-2 drops of tween 20

#### **MS medium** (for 1l)

|                                  |         |
|----------------------------------|---------|
| Murashige and Skoog dried medium | 4.41g   |
| Sucrose 3%                       | 30g     |
| Agar (0.8%)                      | 8g      |
| Water                            | upto 1l |

#### **Extraction buffer** (for 100ml)

|                        |        |
|------------------------|--------|
| 0.1M Tris-HCl (pH 7.5) | 10ml   |
| 0.05M EDTA (pH 8.0)    | 10ml   |
| 10% SDS                | 12.5ml |
| Water                  | 67.5ml |

#### **6M ammonium acetate** (NH<sub>4</sub>C<sub>2</sub>H<sub>3</sub>O<sub>2</sub>) (for 50ml)

|                                                                 |         |
|-----------------------------------------------------------------|---------|
| 6M NH <sub>4</sub> C <sub>2</sub> H <sub>3</sub> O <sub>2</sub> | 23.124g |
| Water                                                           | 50ml    |

Store at 4°C

#### **SS DNA** (140µg/ml) (for 50ml)

|                  |        |
|------------------|--------|
| 50X TAE (pH 6.3) | 5ml    |
| SS DNA (10mg/ml) | 0.7ml  |
| Water            | 44.3ml |

Store at 4°C

#### **3M Sodium Acetate** (NaOAc, pH 5.2) (for 100ml)

|                              |       |
|------------------------------|-------|
| Sodium acetate (m.w.-136.08) | 40.8g |
|------------------------------|-------|

|       |      |
|-------|------|
| Water | 70ml |
|-------|------|

Adjust pH to 5.2

|       |            |
|-------|------------|
| Water | upto 100ml |
|-------|------------|

Sterilise the solution by 0.22 µm membrane filter

**10 x T.E.**

100mM Tris

10mM EDTA

pH7.5

**1 x T.E.**

|        |      |
|--------|------|
| 10xT.E | 10ml |
| Water  | 90ml |

**5x Citric Buffer** (for 50ml)

|                       |        |
|-----------------------|--------|
| Water                 | 30ml   |
| Sodium Citrate (50mM) | 0.735g |
| 0.5M EDTA (50mM)      | 5ml    |

Adjust pH5.5 using Citric acid powder, add water upto 50ml, filter solution and stored at room temperature

**1x Citric Buffer** (for 100ml)

|                  |      |
|------------------|------|
| 5x Citric Buffer | 20ml |
| Water            | 80ml |

**20x SSC** (for 1l)

|                |         |
|----------------|---------|
| NaCl           | 175.3g  |
| Sodium citrate | 88.2g   |
| pH             | 7.0     |
| Water          | upto 1l |

**2x SSC** (for 1l)

|         |        |
|---------|--------|
| 20x SSC | 100mls |
| Water   | 900mls |

**Hybridisation solution** (HS, for 1l)

|                   |                   |                  |
|-------------------|-------------------|------------------|
| Formamide         | 500 µl (for FISH) | 650µl (for GISH) |
| 20XSSC            | 100µl             |                  |
| dextranesulphate  | 200µl             |                  |
| dH <sub>2</sub> O | 200µl (for FISH)  | 50µl (for GISH)  |

**4x SSC with added Tween** (for 1l)

|         |        |
|---------|--------|
| 20x SSC | 200mls |
| Tween   | 2mls   |
| Water   | 798mls |

**DAPI** (4, 6-diamidino-2-phenylindole)

Stock: 100µg/ml DAPI in water (store at 20°C)

Use: 2 µg/ml diluted in PBS

**10X PBS** (phosphate buffered saline, for 1l)

|                                  |       |
|----------------------------------|-------|
| NaCl                             | 80g   |
| KCl                              | 2g    |
| Na <sub>2</sub> HPO <sub>4</sub> | 14.4g |
| KH <sub>2</sub> PO <sub>4</sub>  | 2.4g  |
| dH <sub>2</sub> O                | 1L    |

Autoclave for 20 minutes

**1x TAE buffer**

|           |      |
|-----------|------|
| Tris base | 40mM |
| Acetate   | 20mM |
| EDTA      | 1mM  |
| pH        | 8.6  |

## Appendix 2

### Backcross individuals selected for genotyping

| S. No. | BC1      | BC2      |          | BC2F1        | BC2F2      | BC3      |          | BC3F1      | BC4      |
|--------|----------|----------|----------|--------------|------------|----------|----------|------------|----------|
| 1      | BC1-113A | BC2-258A | BC2-444D | BC2F1-201A   | BC2F2-120A | BC3-100A | BC3-423F | BC3F1-138A | BC4-114A |
| 2      | BC1-113B | BC2-258B | BC2-444E | BC2F1-207A   | BC2F2-120B | BC3-100C | BC3-423G | BC3F1-138B | BC4-114B |
| 3      | BC1-113C | BC2-258C | BC2-537A | BC2F1-210A   | BC2F2-121A | BC3-100D | BC3-423H | BC3F1-138C | BC4-115A |
| 4      | BC1-114A | BC2-259A | BC2-538A | BC2F1-210B   | BC2F2-121B | BC3-100E | BC3-427A | BC3F1-138D | BC4-116A |
| 5      | BC1-114B | BC2-259B | BC2-538B | BC2F1-210C   | BC2F2-122A | BC3-100F | BC3-434A | BC3F1-141A | BC4-131A |
| 6      | BC1-115A | BC2-259C | BC2-538C | BC2F1-210D   | BC2F2-122B | BC3-101A | BC3-435A | BC3F1-141B | BC4-131B |
| 7      | BC1-115B | BC2-260A | BC2-539A | BC2F1-213A   | BC2F2-123A | BC3-101B | BC3-435B | BC3F1-141C | BC4-131C |
| 8      | BC1-190  | BC2-260B | BC2-539B | BC2F1-216A   | BC2F2-123B | BC3-101C | BC3-436A | BC3F1-141D | BC4-131D |
| 9      | BC1-192A | BC2-261A | BC2-539C | BC2F1-248-1  | BC2F2-124A | BC3-102A | BC3-437A | BC3F1-141E | BC4-132A |
| 10     | BC1-192B | BC2-262A | BC2-539D | BC2F1-248-2  | BC2F2-124B | BC3-103A | BC3-437B | BC3F1-141F | BC4-132B |
| 11     | BC1-194  | BC2-262B | BC2-540A | BC2F1-248-3  | BC2F2-125A | BC3-103C | BC3-437C | BC3F1-141G | BC4-132C |
| 12     | BC1-195  | BC2-262C | BC2-541A | BC2F1-248-4  | BC2F2-125B | BC3-103D | BC3-437D | BC3F1-141H | BC4-132D |
| 13     | BC1-243  | BC2-263A | BC2-542A | BC2F1-248-5  | BC2F2-126A | BC3-104A | BC3-437E | BC3F1-141I | BC4-132E |
| 14     | BC1-300A | BC2-263B | BC2-542C | BC2F1-248-6  | BC2F2-126B | BC3-104B | BC3-437F | BC3F1-141J | BC4-132F |
| 15     | BC1-300B | BC2-263C | BC2-543A | BC2F1-248-7  | BC2F2-127A | BC3-104C | BC3-437G | BC3F1-141K | BC4-132G |
| 16     | BC1-300C | BC2-264A | BC2-543B | BC2F1-248-8  | BC2F2-127B | BC3-105A | BC3-437H | BC3F1-141L | BC4-136A |
| 17     | BC1-302  | BC2-264B | BC2-543C | BC2F1-249-1  | BC2F2-128C | BC3-105B | BC3-437I | BC3F1-143A | BC4-136B |
| 18     | BC1-303  | BC2-264C | BC2-544A | BC2F1-249-10 | BC2F2-230A | BC3-106A | BC3-439A | BC3F1-143B | BC4-136C |
| 19     | BC1-304  | BC2-265A | BC2-545A | BC2F1-249-11 | BC2F2-230B | BC3-106C | BC3-439B | BC3F1-143C | BC4-136D |
| 20     | BC1-384A | BC2-265B | BC2-546C | BC2F1-249-2  | BC2F2-231A | BC3-106E | BC3-439C | BC3F1-143D | BC4-144A |
| 21     | BC1-384B | BC2-265D | BC2-547A | BC2F1-249-3  | BC2F2-231B | BC3-106F | BC3-439D | BC3F1-143E | BC4-144B |
| 22     | BC1-384C | BC2-265E | BC2-548A | BC2F1-249-4  | BC2F2-232A | BC3-107A | BC3-439E | BC3F1-143F | BC4-144C |
| 23     | BC1-386A | BC2-265F | BC2-548B | BC2F1-249-5  | BC2F2-232B | BC3-108A | BC3-439F | BC3F1-143G | BC4-144D |



|    |          |          |          |             |            |          |          |            |          |
|----|----------|----------|----------|-------------|------------|----------|----------|------------|----------|
| 24 | BC1-387A | BC2-266A | BC2-548C | BC2F1-249-6 | BC2F2-233A | BC3-109A | BC3-439G | BC3F1-143H | BC4-144E |
| 25 | BC1-387B | BC2-266B | BC2-548D | BC2F1-249-7 | BC2F2-233B | BC3-110A | BC3-439H | BC3F1-145A | BC4-144F |
| 26 | BC1-388A | BC2-266C | BC2-549A | BC2F1-249-8 | BC2F2-254A | BC3-111A | BC3-440A | BC3F1-145B | BC4-144G |
| 27 | BC1-389A | BC2-267A | BC2-549B | BC2F1-249-9 | BC2F2-254B | BC3-112A | BC3-440B | BC3F1-145C | BC4-144H |
| 28 | BC1-389B | BC2-267B | BC2-549C |             | BC2F2-255A | BC3-113A | BC3-440C | BC3F1-145D | BC4-146A |
| 29 | BC1-390A | BC2-267C | BC2-549D |             | BC2F2-255B | BC3-234A | BC3-441A | BC3F1-145G | BC4-146B |
| 30 | BC1-392A | BC2-268A | BC2-550A |             | BC2F2-256A | BC3-234B | BC3-441B | BC3F1-145H | BC4-146C |
| 31 | BC1-393A | BC2-268B | BC2-550C |             | BC2F2-256B | BC3-235A | BC3-441C | BC3F1-145I | BC4-146D |
| 32 | BC1-395A | BC2-269A | BC2-550D |             | BC2F2-258A | BC3-235B | BC3-441D | BC3F1-147A | BC4-146E |
| 33 | BC1-395B | BC2-269B | BC2-551A |             | BC2F2-258B | BC3-236B | BC3-441E | BC3F1-147B | BC4-146F |
| 34 | BC1-396A | BC2-269C | BC2-551B |             | BC2F2-260A | BC3-271A | BC3-442A | BC3F1-147C |          |
| 35 | BC1-397A | BC2-270A | BC2-551C |             | BC2F2-260B | BC3-271B | BC3-442B | BC3F1-147D |          |
| 36 |          | BC2-271A | BC2-552A |             | BC2F2-260C | BC3-272A | BC3-442C | BC3F1-149A |          |
| 37 |          | BC2-271B | BC2-573A |             | BC2F2-261A | BC3-272B | BC3-442D | BC3F1-149B |          |
| 38 |          | BC2-271C | BC2-573B |             | BC2F2-261B | BC3-272C | BC3-447A | BC3F1-149C |          |
| 39 |          | BC2-272A | BC2-575A |             | BC2F2-298A | BC3-273A | BC3-447B | BC3F1-149D |          |
| 40 |          | BC2-275A | BC2-575B |             | BC2F2-298B | BC3-274A | BC3-447C | BC3F1-149E |          |
| 41 |          | BC2-275D | BC2-575D |             | BC2F2-299A | BC3-367A | BC3-447D | BC3F1-149F |          |
| 42 |          | BC2-275E | BC2-576A |             | BC2F2-299B | BC3-367B | BC3-447E | BC3F1-149G |          |
| 43 |          | BC2-276A | BC2-577B |             | BC2F2-300A | BC3-368A | BC3-447F | BC3F1-149H |          |
| 44 |          | BC2-276B | BC2-578B |             | BC2F2-300B | BC3-368B | BC3-447G | BC3F1-150A |          |
| 45 |          | BC2-276C | BC2-578C |             | BC2F2-301A | BC3-368C | BC3-447H | BC3F1-150B |          |
| 46 |          | BC2-359A | BC2-578D |             | BC2F2-301B | BC3-369C | BC3-451C | BC3F1-150C |          |
| 47 |          | BC2-360A | BC2-579A |             | BC2F2-302A | BC3-369D | BC3-452A | BC3F1-150D |          |
| 48 |          | BC2-360B | BC2-579B |             | BC2F2-302B | BC3-370A | BC3-452B | BC3F1-150E |          |
| 49 |          | BC2-361A | BC2-579C |             | BC2F2-303A | BC3-371A | BC3-452C | BC3F1-150G |          |
| 50 |          | BC2-362A | BC2-580A |             | BC2F2-303B | BC3-371B | BC3-452F | BC3F1-150H |          |
| 51 |          | BC2-362B | BC2-580B |             | BC2F2-304A | BC3-372A | BC3-472A | BC3F1-151A |          |
| 52 |          | BC2-363A | BC2-635A |             | BC2F2-304B | BC3-372B | BC3-472B | BC3F1-151B |          |

|              |           |            |          |           |            |            |          |            |           |
|--------------|-----------|------------|----------|-----------|------------|------------|----------|------------|-----------|
| 53           |           | BC2-364A   | BC2-635B |           | BC2F2-305A | BC3-373A   | BC3-474A | BC3F1-151C |           |
| 54           |           | BC2-365A   | BC2-636A |           | BC2F2-306A | BC3-373B   | BC3-474B | BC3F1-151E |           |
| 55           |           | BC2-366A   | BC2-636B |           | BC2F2-306B | BC3-374A   | BC3-477A | BC3F1-151F |           |
| 56           |           | BC2-366B   | BC2-637A |           | BC2F2-307A | BC3-374B   | BC3-477C | BC3F1-151I |           |
| 57           |           | BC2-367A   | BC2-637B |           | BC2F2-307B | BC3-375A   | BC3-477E | BC3F1-152A |           |
| 58           |           | BC2-368A   | BC2-637C |           |            | BC3-375B   | BC3-91A  | BC3F1-152B |           |
| 59           |           | BC2-368B   | BC2-702A |           |            | BC3-376A   | BC3-92A  | BC3F1-152C |           |
| 60           |           | BC2-369A   | BC2-703A |           |            | BC3-376B   | BC3-93A  | BC3F1-152D |           |
| 61           |           | BC2-371A   | BC2-703B |           |            | BC3-377A   | BC3-93B  | BC3F1-152E |           |
| 62           |           | BC2-371B   |          |           |            | BC3-377B   | BC3-93C  | BC3F1-152F |           |
| 63           |           | BC2-372A   |          |           |            | BC3-378A   | BC3-94A  | BC3F1-152I |           |
| 64           |           | BC2-372B   |          |           |            | BC3-378B   | BC3-94B  | BC3F1-152J |           |
| 65           |           | BC2-374A   |          |           |            | BC3-419A   | BC3-94C  | BC3F1-152K |           |
| 66           |           | BC2-375A   |          |           |            | BC3-420A   | BC3-95A  | BC3F1-154A |           |
| 67           |           | BC2-375B   |          |           |            | BC3-420B   | BC3-96A  | BC3F1-154B |           |
| 68           |           | BC2-443A   |          |           |            | BC3-422A   | BC3-97A  | BC3F1-154C |           |
| 69           |           | BC2-443B   |          |           |            | BC3-422B   | BC3-98A  | BC3F1-154D |           |
| 70           |           | BC2-443C   |          |           |            | BC3-423B   | BC3-98D  | BC3F1-154E |           |
| 71           |           | BC2-444A   |          |           |            | BC3-423C   | BC3-98E  | BC3F1-154F |           |
| 72           |           | BC2-444B   |          |           |            | BC3-423D   | BC3-99A  | BC3F1-154G |           |
| 73           |           | BC2-444C   |          |           |            | BC3-423E   |          | BC3F1-154H |           |
| <b>Total</b> | <b>35</b> | <b>134</b> |          | <b>27</b> | <b>57</b>  | <b>145</b> |          | <b>73</b>  | <b>33</b> |

### Appendix 3

#### Markers of the A genome of *T. timopheevii* with cM distance

| Chr1At      | cM    | Chr2At      | cM    | Chr3At      | cM    | Chr4At      | cM     | Chr5At      | cM     | Chr6At      | cM    | Chr7At      | cM     |
|-------------|-------|-------------|-------|-------------|-------|-------------|--------|-------------|--------|-------------|-------|-------------|--------|
| AX-94730241 | 0     | AX-95129278 | 0     | AX-94598356 | 0     | AX-94857918 | 0      | AX-94569085 | 0      | AX-94641398 | 0     | AX-94700184 | 0      |
| AX-94393003 | 0     | AX-94992596 | 0     | AX-94954492 | 1.818 | AX-94771772 | 3.068  | AX-94538890 | 0      | AX-95241582 | 0     | AX-95122633 | 3.92   |
| AX-94503609 | 0.199 | AX-94898093 | 0     | AX-95207862 | 2.017 | AX-94690629 | 7.636  | AX-94426799 | 0      | AX-94948332 | 0     | AX-94609778 | 6.777  |
| AX-95014575 | 0.199 | AX-94872303 | 0     | AX-95206184 | 2.216 | AX-94856207 | 7.835  | AX-94961078 | 0.398  | AX-86179112 | 0     | AX-94899428 | 9.846  |
| AX-95151422 | 0.199 | AX-94618443 | 5.666 | AX-94654157 | 8.329 | AX-94568929 | 7.835  | AX-95109520 | 4.749  | AX-94706334 | 0     | AX-86172286 | 13.338 |
| AX-94774946 | 0.199 | AX-94634892 | 6.668 | AX-94995928 | 8.329 | AX-94397930 | 8.034  | AX-94923920 | 6.567  | AX-95144385 | 0     | AX-94704678 | 13.736 |
| AX-94522187 | 0.199 | AX-94976126 | 7.468 | AX-94557165 | 8.329 | AX-94862616 | 10.058 | AX-94415346 | 10.918 | AX-94600202 | 2.232 | AX-94407354 | 13.935 |
| AX-94673150 | 0.199 | AX-95681996 | 14.03 | AX-86178969 | 8.329 | AX-94945797 | 10.058 | AX-95248126 | 10.918 | AX-86179538 | 2.43  | AX-94533562 | 14.534 |
| AX-94732962 | 0.199 | AX-94740707 | 15.85 | AX-89536025 | 8.329 | AX-94472289 | 10.858 | AX-94456236 | 17.936 | AX-95021534 | 2.43  | AX-94836900 | 20.423 |

|             |           |             |           |             |           |             |            |             |            |             |       |             |            |
|-------------|-----------|-------------|-----------|-------------|-----------|-------------|------------|-------------|------------|-------------|-------|-------------|------------|
| AX-94922849 | 0.19<br>9 | AX-94539132 | 16.6<br>5 | AX-94702169 | 8.32<br>9 | AX-94479623 | 11.25<br>7 | AX-94899135 | 19.96      | AX-94625175 | 2.43  | AX-94683427 | 24.99<br>1 |
| AX-86173170 | 0.19<br>9 | AX-94989868 | 16.6<br>5 | AX-94397528 | 8.32<br>9 | AX-95190358 | 11.25<br>7 | AX-94846190 | 28.83<br>9 | AX-94668789 | 2.43  | AX-95252450 | 25.59      |
| AX-94594465 | 0.19<br>9 | AX-95168254 | 19.3      | AX-94434800 | 8.92<br>8 | AX-94855600 | 11.25<br>7 | AX-95175117 | 29.23<br>7 | AX-95251480 | 2.43  | AX-95080368 | 25.59      |
| AX-94526116 | 0.19<br>9 | AX-94459270 | 19.3      | AX-95024395 | 8.92<br>8 | AX-94669734 | 11.25<br>7 | AX-95097918 | 29.23<br>7 | AX-94445103 | 2.43  | AX-94383872 | 26.18<br>8 |
| AX-94700819 | 0.19<br>9 | AX-94427576 | 19.5      | AX-94641244 | 8.92<br>8 | AX-94507064 | 11.25<br>7 | AX-94945628 | 35.57<br>5 | AX-95236394 | 2.43  | AX-95198768 | 28.83<br>7 |
| AX-94700094 | 1.60<br>7 | AX-94470676 | 19.5      | AX-94927871 | 9.52<br>6 | AX-94576066 | 11.25<br>7 | AX-94430309 | 36.17<br>4 | AX-94902458 | 2.629 | AX-94529333 | 29.23<br>5 |
| AX-95106105 | 1.60<br>7 | AX-94921756 | 19.9      | AX-94523332 | 9.52<br>6 | AX-94940770 | 13.07<br>5 | AX-94603374 | 38.61<br>3 | AX-95225835 | 2.629 | AX-94406566 | 31.46<br>7 |
| AX-94493329 | 2.40<br>7 | AX-94818415 | 21.9<br>2 | AX-94542938 | 9.52<br>6 | AX-94476473 | 13.27<br>4 | AX-94651790 | 40.43<br>2 | AX-94835245 | 2.629 | AX-94703949 | 31.86<br>5 |
| AX-95146445 | 2.40<br>7 | AX-95169440 | 22.5<br>2 | AX-94466419 | 9.52<br>6 | AX-94721591 | 13.67<br>2 | AX-95076236 | 40.63      | AX-94639519 | 2.629 | AX-94880546 | 32.06<br>4 |
| AX-94777342 | 2.60<br>6 | AX-94464916 | 22.5<br>2 | AX-94448019 | 9.52<br>6 | AX-94660240 | 13.67<br>2 | AX-94723512 | 41.43      | AX-94881038 | 9.193 | AX-94491164 | 32.06<br>4 |

|             |           |             |           |             |           |             |            |             |            |             |            |             |            |
|-------------|-----------|-------------|-----------|-------------|-----------|-------------|------------|-------------|------------|-------------|------------|-------------|------------|
| AX-94629293 | 3.00<br>5 | AX-94394578 | 23.5<br>2 | AX-95003784 | 9.52<br>6 | AX-94686603 | 13.67<br>2 | AX-94891640 | 41.43      | AX-94568819 | 9.193      | AX-94938968 | 32.06<br>4 |
| AX-94917775 | 3.00<br>5 | AX-94636452 | 23.7<br>2 | AX-95220838 | 9.52<br>6 | AX-94942459 | 13.67<br>2 | AX-94439832 | 49.37      | AX-94577446 | 10.19<br>5 | AX-94542816 | 32.06<br>4 |
| AX-94384347 | 3.00<br>5 | AX-95183011 | 24.9<br>3 | AX-95245143 | 9.52<br>6 | AX-94832412 | 14.07<br>1 | AX-94749230 | 49.96<br>9 | AX-94786019 | 10.39<br>3 | AX-94383516 | 32.06<br>4 |
| AX-95069046 | 3.00<br>5 | AX-94460418 | 27.3<br>6 | AX-94783547 | 9.52<br>6 | AX-95144713 | 14.07<br>1 | AX-94903992 | 49.96<br>9 | AX-94424299 | 10.39<br>3 | AX-95172377 | 32.06<br>4 |
| AX-94439447 | 3.00<br>5 | AX-94657384 | 28.9<br>8 | AX-94502353 | 9.52<br>6 | AX-94387796 | 14.26<br>9 | AX-94392984 | 50.76<br>9 | AX-95197676 | 10.39<br>3 | AX-94502972 | 32.06<br>4 |
| AX-94714481 | 3.00<br>5 | AX-95188312 | 30.1<br>8 | AX-94852544 | 9.52<br>6 | AX-94529621 | 14.26<br>9 | AX-94419595 | 51.36<br>8 | AX-95199148 | 10.39<br>3 | AX-94599101 | 32.66<br>3 |
| AX-94713673 | 3.20<br>3 | AX-95131782 | 30.1<br>8 | AX-94393196 | 9.52<br>6 | AX-94492884 | 14.26<br>9 | AX-94399337 | 54.64<br>7 | AX-94910882 | 10.39<br>3 | AX-94494916 | 34.48<br>1 |
| AX-94731169 | 3.20<br>3 | AX-94697318 | 30.5<br>8 | AX-94709618 | 9.52<br>6 | AX-94554728 | 14.46<br>8 | AX-95176087 | 54.64<br>7 | AX-94459673 | 10.39<br>3 | AX-86171357 | 34.48<br>1 |
| AX-94456521 | 3.20<br>3 | AX-94666225 | 31.3<br>8 | AX-94548447 | 9.52<br>6 | AX-94430995 | 14.46<br>8 | AX-94773269 | 58.35<br>3 | AX-94746124 | 10.39<br>3 | AX-94986725 | 34.48<br>1 |
| AX-95207074 | 3.60<br>2 | AX-95163698 | 33.6<br>1 | AX-94948041 | 9.52<br>6 | AX-94422746 | 14.66<br>7 | AX-94748288 | 58.35<br>3 | AX-94619757 | 10.39<br>3 | AX-94630072 | 34.48<br>1 |

|             |           |             |           |             |           |             |            |             |            |             |            |             |            |
|-------------|-----------|-------------|-----------|-------------|-----------|-------------|------------|-------------|------------|-------------|------------|-------------|------------|
| AX-94993961 | 3.80<br>1 | AX-94504198 | 34.4<br>1 | AX-94823108 | 9.52<br>6 | AX-94735128 | 15.26<br>6 | AX-94785241 | 58.35<br>3 | AX-94891053 | 10.99<br>2 | AX-94689244 | 34.48<br>1 |
| AX-94870541 | 3.99<br>9 | AX-94896111 | 34.4<br>1 | AX-94612798 | 9.52<br>6 | AX-94945870 | 15.26<br>6 | AX-94557327 | 58.95<br>2 | AX-94674861 | 11.39<br>1 | AX-95215247 | 34.68      |
| AX-94490465 | 4.79<br>9 | AX-94751668 | 34.4<br>1 | AX-94427362 | 9.52<br>6 | AX-94663483 | 15.66<br>4 | AX-94691847 | 59.95<br>4 | AX-94971944 | 11.39<br>1 | AX-94850011 | 34.68      |
| AX-94400275 | 4.79<br>9 | AX-95008724 | 34.4<br>1 | AX-94471182 | 9.52<br>6 | AX-94578210 | 16.06<br>3 | AX-95095532 | 60.75<br>4 | AX-94433552 | 11.58<br>9 | AX-95115516 | 34.68      |
| AX-94411374 | 4.79<br>9 | AX-94412799 | 34.6<br>1 | AX-94946799 | 9.52<br>6 | AX-86179203 | 16.06<br>3 | AX-94494261 | 60.75<br>4 | AX-94776871 | 11.58<br>9 | AX-94695508 | 34.68      |
| AX-95182380 | 5.19<br>8 | AX-94528590 | 34.6<br>1 | AX-95157963 | 9.52<br>6 | AX-86169765 | 16.06<br>3 | AX-94489770 | 60.75<br>4 | AX-94563888 | 12.59<br>1 | AX-94442494 | 34.87<br>9 |
| AX-94905532 | 5.19<br>8 | AX-94906814 | 34.6<br>1 | AX-94617990 | 9.52<br>6 | AX-94814284 | 16.06<br>3 | AX-94381965 | 61.55<br>4 | AX-94618325 | 12.79      | AX-95130767 | 34.87<br>9 |
| AX-94939314 | 5.19<br>8 | AX-86165901 | 35.8<br>2 | AX-94710954 | 9.52<br>6 |             |            | AX-94429098 | 62.35<br>4 | AX-94488716 | 12.79      | AX-94422615 | 34.87<br>9 |
| AX-94778336 | 7.63<br>7 | AX-94853843 | 36.0<br>2 | AX-94960174 | 9.52<br>6 |             |            | AX-94483629 | 63.15<br>4 | AX-94468806 | 12.79      | AX-94437464 | 34.87<br>9 |
| AX-94528863 | 7.83<br>6 | AX-94396146 | 36.8<br>2 | AX-94690364 | 9.52<br>6 |             |            | AX-94622175 | 63.55<br>2 | AX-95189832 | 13.79<br>2 | AX-94595599 | 34.87<br>9 |

|                 |           |                 |           |                 |           |  |  |                 |            |                 |            |                 |            |
|-----------------|-----------|-----------------|-----------|-----------------|-----------|--|--|-----------------|------------|-----------------|------------|-----------------|------------|
| AX-<br>94619761 | 7.83<br>6 | AX-<br>94443516 | 36.8<br>2 | AX-<br>94598003 | 9.52<br>6 |  |  | AX-<br>94398500 | 67.04<br>4 | AX-<br>95630343 | 13.99<br>1 | AX-<br>94389087 | 34.87<br>9 |
| AX-<br>94693971 | 8.23<br>5 | AX-<br>95184497 | 37.0<br>1 | AX-<br>95130498 | 9.52<br>6 |  |  | AX-<br>95250267 | 67.84<br>4 | AX-<br>86177686 | 13.99<br>1 | AX-<br>95079348 | 34.87<br>9 |
| AX-<br>94684631 | 8.23<br>5 | AX-<br>94828061 | 37.2<br>1 | AX-<br>95097082 | 9.52<br>6 |  |  | AX-<br>94445171 | 68.84<br>6 | AX-<br>94556575 | 13.99<br>1 | AX-<br>94391320 | 34.87<br>9 |
| AX-<br>94478936 | 8.43<br>3 | AX-<br>95223335 | 37.2<br>1 | AX-<br>94788613 | 9.52<br>6 |  |  | AX-<br>94452971 | 71.91<br>5 | AX-<br>95025699 | 14.19      | AX-<br>94538964 | 34.87<br>9 |
| AX-<br>94620755 | 10.0<br>5 | AX-<br>95115213 | 37.2<br>1 | AX-<br>94381828 | 9.52<br>6 |  |  | AX-<br>94905515 | 72.71<br>5 | AX-<br>95630344 | 14.78<br>9 | AX-<br>94839659 | 34.87<br>9 |
| AX-<br>94978926 | 11.2<br>5 | AX-<br>94911937 | 37.6<br>1 | AX-<br>94463669 | 9.52<br>6 |  |  |                 |            | AX-<br>95130067 | 16.60<br>7 | AX-<br>94779311 | 34.87<br>9 |
| AX-<br>94530188 | 15.1<br>7 | AX-<br>94727794 | 38.6<br>1 | AX-<br>94428990 | 9.52<br>6 |  |  |                 |            | AX-<br>94500374 | 19.04<br>7 | AX-<br>94976796 | 35.07<br>7 |
| AX-<br>94721225 | 15.3<br>7 | AX-<br>94742725 | 38.6<br>1 | AX-<br>94422817 | 9.52<br>6 |  |  |                 |            | AX-<br>94896372 | 21.90<br>5 | AX-<br>94417359 | 35.07<br>7 |
| AX-<br>94590927 | 16.9<br>8 | AX-<br>94678573 | 38.6<br>1 | AX-<br>95182054 | 9.52<br>6 |  |  |                 |            | AX-<br>94811377 | 23.92<br>9 | AX-<br>94505780 | 35.67<br>6 |
| AX-<br>94498696 | 17.3<br>8 | AX-<br>95167337 | 38.8<br>1 | AX-<br>94520083 | 9.52<br>6 |  |  |                 |            | AX-<br>94551877 | 25.74<br>7 | AX-<br>94387074 | 41.12<br>1 |

|             |           |             |           |             |           |  |  |  |  |             |            |             |            |
|-------------|-----------|-------------|-----------|-------------|-----------|--|--|--|--|-------------|------------|-------------|------------|
| AX-95180111 | 19.8<br>2 | AX-94799340 | 38.8<br>1 | AX-94487190 | 9.52<br>6 |  |  |  |  | AX-94494894 | 26.34<br>6 | AX-94638909 | 41.12<br>1 |
| AX-94990974 | 22.4<br>7 | AX-94629399 | 38.8<br>1 | AX-94861433 | 9.52<br>6 |  |  |  |  | AX-94800440 | 32.23<br>5 | AX-94602262 | 43.35<br>3 |
| AX-94518993 | 25.1<br>2 | AX-95072207 | 38.8<br>1 | AX-95630046 | 9.52<br>6 |  |  |  |  | AX-94395967 | 32.23<br>5 | AX-94486555 | 43.35<br>3 |
| AX-94999281 | 25.7<br>2 | AX-94638064 | 38.8<br>1 | AX-94398068 | 9.52<br>6 |  |  |  |  | AX-94486279 | 44.54<br>7 | AX-95120206 | 43.95<br>1 |
| AX-94426657 | 26.3<br>2 | AX-94462589 | 39.6<br>1 | AX-94928542 | 9.52<br>6 |  |  |  |  | AX-94951345 | 49.55<br>1 | AX-94912175 | 45.56<br>4 |
| AX-94403165 | 26.5<br>1 | AX-95257613 | 40.4<br>1 | AX-95216392 | 9.52<br>6 |  |  |  |  |             |            | AX-94522885 | 45.56<br>4 |
| AX-94667493 | 26.5<br>1 | AX-94538364 | 40.6<br>1 | AX-95123927 | 9.52<br>6 |  |  |  |  |             |            | AX-94384726 | 46.36<br>4 |
| AX-94476277 | 26.5<br>1 | AX-95176938 | 42.4<br>3 | AX-94852279 | 9.52<br>6 |  |  |  |  |             |            | AX-95245897 | 46.36<br>4 |
| AX-94434430 | 30.6<br>5 | AX-95192332 | 42.8<br>3 | AX-94495556 | 9.52<br>6 |  |  |  |  |             |            | AX-89640006 | 46.36<br>4 |
| AX-94436223 | 33.7<br>2 | AX-95073291 | 42.8<br>3 | AX-95234197 | 9.52<br>6 |  |  |  |  |             |            | AX-94470543 | 46.36<br>4 |



|                 |           |                 |           |                 |           |  |  |  |  |  |  |                 |            |
|-----------------|-----------|-----------------|-----------|-----------------|-----------|--|--|--|--|--|--|-----------------|------------|
| AX-<br>94400729 | 37.8<br>5 | AX-<br>94804880 | 44.0<br>3 | AX-<br>94541427 | 9.52<br>6 |  |  |  |  |  |  | AX-<br>95112215 | 46.56<br>3 |
| AX-<br>94473649 | 39.2<br>6 | AX-<br>95259382 | 45.8<br>5 | AX-<br>94987223 | 9.52<br>6 |  |  |  |  |  |  | AX-<br>86176910 | 46.56<br>3 |
| AX-<br>95005100 | 45.3<br>7 | AX-<br>95247903 | 47.2<br>6 | AX-<br>94394183 | 9.52<br>6 |  |  |  |  |  |  | AX-<br>94547905 | 50.05<br>5 |
| AX-<br>94982575 | 45.5<br>7 | AX-<br>94388468 | 47.2<br>6 | AX-<br>94586703 | 9.52<br>6 |  |  |  |  |  |  | AX-<br>94481110 | 50.05<br>5 |
| AX-<br>95093988 | 45.5<br>7 | AX-<br>94437829 | 47.6<br>6 | AX-<br>94812241 | 13.2<br>3 |  |  |  |  |  |  | AX-<br>94436934 | 52.08      |
| AX-<br>95202547 | 45.7<br>7 | AX-<br>94700503 | 49.0<br>7 | AX-<br>94568176 | 13.4<br>3 |  |  |  |  |  |  | AX-<br>94381327 | 52.08      |
|                 |           | AX-<br>94916744 | 52.7<br>7 | AX-<br>95163612 | 13.4<br>3 |  |  |  |  |  |  |                 |            |
|                 |           | AX-<br>95225412 | 62.8<br>5 | AX-<br>94961153 | 13.4<br>3 |  |  |  |  |  |  |                 |            |
|                 |           | AX-<br>94929700 | 66.1<br>3 | AX-<br>94771453 | 13.4<br>3 |  |  |  |  |  |  |                 |            |
|                 |           | AX-<br>95250028 | 66.1<br>3 | AX-<br>94493067 | 13.4<br>3 |  |  |  |  |  |  |                 |            |

|  |  |                 |           |                 |           |  |  |  |  |  |  |  |  |
|--|--|-----------------|-----------|-----------------|-----------|--|--|--|--|--|--|--|--|
|  |  | AX-<br>95256835 | 66.1<br>3 | AX-<br>94510964 | 13.4<br>3 |  |  |  |  |  |  |  |  |
|  |  |                 |           | AX-<br>95125002 | 13.4<br>3 |  |  |  |  |  |  |  |  |
|  |  |                 |           | AX-<br>95218076 | 13.4<br>3 |  |  |  |  |  |  |  |  |
|  |  |                 |           | AX-<br>94720408 | 13.8<br>3 |  |  |  |  |  |  |  |  |
|  |  |                 |           | AX-<br>94972830 | 14.0<br>3 |  |  |  |  |  |  |  |  |
|  |  |                 |           | AX-<br>95201257 | 14.0<br>3 |  |  |  |  |  |  |  |  |
|  |  |                 |           | AX-<br>94813670 | 14.0<br>3 |  |  |  |  |  |  |  |  |
|  |  |                 |           | AX-<br>95070910 | 14.6<br>3 |  |  |  |  |  |  |  |  |
|  |  |                 |           | AX-<br>94829627 | 14.6<br>3 |  |  |  |  |  |  |  |  |
|  |  |                 |           | AX-<br>94663948 | 14.8<br>3 |  |  |  |  |  |  |  |  |

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|--|--|--|--|-----------------|-----------|--|--|--|--|--|--|--|--|
|  |  |  |  | AX-<br>94485226 | 15.0<br>2 |  |  |  |  |  |  |  |  |
|  |  |  |  | AX-<br>94552167 | 16.4<br>3 |  |  |  |  |  |  |  |  |
|  |  |  |  | AX-<br>86176361 | 16.4<br>3 |  |  |  |  |  |  |  |  |
|  |  |  |  | AX-<br>94743140 | 16.8<br>3 |  |  |  |  |  |  |  |  |
|  |  |  |  | AX-<br>95207768 | 17.2<br>3 |  |  |  |  |  |  |  |  |
|  |  |  |  | AX-<br>95081840 | 17.2<br>3 |  |  |  |  |  |  |  |  |

### Markers of the G genome of *T. timopheevii* with cM distance

| Chr1G       | cM    | Chr2G       | cM    | Chr3G       | cM    | Chr4G       | cM    | Chr5G       | cM    | Chr6G       | cM    | Chr7G       | cM    |
|-------------|-------|-------------|-------|-------------|-------|-------------|-------|-------------|-------|-------------|-------|-------------|-------|
| AX-94460938 | 0     | AX-94382746 | 0     | AX-94485280 | 0     | AX-89428116 | 0     | AX-95015550 | 0     | AX-94539061 | 0     | AX-94793084 | 0     |
| AX-94645609 | 0     | AX-94725049 | 0     | AX-94887138 | 0     | AX-94757730 | 0.199 | AX-94833651 | 0     | AX-95256407 | 0     | AX-94712159 | 0.199 |
| AX-94711679 | 0     | AX-94698374 | 0     | AX-94493124 | 0     | AX-95256633 | 6.536 | AX-94647555 | 0     | AX-94745890 | 0     | AX-94734619 | 0.199 |
| AX-94419438 | 0     | AX-94426629 | 0     | AX-94785229 | 2.858 | AX-86167869 | 6.735 | AX-95158641 | 0     | AX-94848968 | 1.818 | AX-95205695 | 0.199 |
| AX-95217989 | 5.445 | AX-95080496 | 0.599 | AX-95630213 | 2.858 | AX-95162453 | 6.735 | AX-94637263 | 0     | AX-95102445 | 2.017 | AX-94836356 | 2.638 |
| AX-95090046 | 5.445 | AX-95138160 | 7.846 | AX-95248822 | 2.858 | AX-94681360 | 7.535 | AX-95141179 | 0     | AX-94905905 | 3.222 | AX-94657427 | 2.638 |
| AX-95095276 | 5.445 | AX-94463791 | 8.044 | AX-94824926 | 2.858 | AX-95241638 | 7.535 | AX-95210844 | 0     | AX-94626439 | 3.222 | AX-94711978 | 2.638 |
| AX-94884435 | 5.445 | AX-94594229 | 8.443 | AX-94728995 | 6.777 | AX-95130149 | 7.535 | AX-94610861 | 0     | AX-95151188 | 3.421 | AX-95223512 | 2.837 |
| AX-95166606 | 5.445 | AX-95205778 | 8.443 | AX-94843732 | 6.976 | AX-95232028 | 7.535 | AX-95196211 | 0.398 | AX-95240811 | 3.421 | AX-95159346 | 3.036 |

|             |       |             |        |             |       |             |       |             |       |             |       |             |       |
|-------------|-------|-------------|--------|-------------|-------|-------------|-------|-------------|-------|-------------|-------|-------------|-------|
| AX-95183541 | 5.445 | AX-95259954 | 8.443  | AX-95222926 | 7.175 | AX-94589541 | 7.535 | AX-95141719 | 0.597 | AX-95142186 | 5.86  | AX-95190773 | 3.434 |
| AX-94843298 | 5.445 | AX-95214925 | 8.841  | AX-94536828 | 7.175 | AX-94663013 | 7.535 | AX-95179297 | 0.597 | AX-94414937 | 7.885 | AX-94441118 | 5.047 |
| AX-94477162 | 5.445 | AX-94896890 | 9.24   | AX-94575738 | 7.175 | AX-94744972 | 7.535 | AX-94800205 | 0.597 | AX-95122886 | 7.885 | AX-94608702 | 5.646 |
| AX-94917253 | 5.445 | AX-94979809 | 9.638  | AX-94987336 | 7.175 | AX-95005286 | 7.535 | AX-95104017 | 0.597 | AX-95010034 | 10.32 | AX-94973270 | 5.646 |
| AX-94645635 | 5.445 | AX-95194288 | 9.638  | AX-94527536 | 7.175 | AX-94889338 | 7.535 | AX-94438942 | 0.597 | AX-94983443 | 10.32 | AX-94424622 | 9.138 |
| AX-95259499 | 5.445 | AX-94547484 | 13.773 | AX-94445008 | 7.175 | AX-94615215 | 7.535 | AX-94596869 | 0.597 | AX-94973992 | 10.32 | AX-94444153 | 9.138 |
| AX-95180246 | 5.445 | AX-95204758 | 18.123 | AX-95251983 | 7.175 | AX-94913400 | 7.535 | AX-94644008 | 0.597 | AX-95198819 | 10.32 | AX-94929921 | 9.138 |
| AX-94598055 | 5.445 | AX-94968580 | 34.12  | AX-95121108 | 7.175 | AX-94995879 | 7.535 | AX-94797492 | 0.597 | AX-95071694 | 10.52 | AX-94574308 | 9.138 |
| AX-94463026 | 5.445 | AX-94487424 | 34.12  | AX-94430479 | 7.175 | AX-94519609 | 7.535 | AX-94643290 | 0.597 | AX-95070251 | 10.72 | AX-94446908 | 9.138 |
| AX-94434696 | 8.725 | AX-94465247 | 34.12  | AX-94907804 | 7.175 | AX-94646391 | 7.535 | AX-94682707 | 0.597 | AX-94587365 | 10.72 | AX-94485553 | 9.138 |

|             |       |             |        |             |       |             |       |             |       |             |       |             |       |
|-------------|-------|-------------|--------|-------------|-------|-------------|-------|-------------|-------|-------------|-------|-------------|-------|
| AX-95106563 | 8.923 | AX-94497304 | 62.449 | AX-95200519 | 7.175 | AX-94797205 | 7.535 | AX-94609579 | 0.597 | AX-95075504 | 10.72 | AX-94534396 | 9.138 |
| AX-94552873 | 8.923 | AX-95225623 | 62.449 | AX-94900033 | 7.175 | AX-95005995 | 7.535 | AX-95161666 | 0.597 | AX-94935446 | 10.72 | AX-94517409 | 9.138 |
| AX-94970442 | 8.923 | AX-94802300 | 62.449 | AX-94703979 | 7.175 | AX-94401780 | 7.535 | AX-94996942 | 0.597 | AX-94830259 | 10.72 | AX-94477496 | 9.138 |
| AX-94418372 | 8.923 | AX-94463522 | 62.449 | AX-95241723 | 7.175 | AX-94834923 | 7.535 | AX-94820343 | 0.597 | AX-94666641 | 10.72 | AX-95183267 | 9.138 |
| AX-94657479 | 8.923 | AX-94585204 | 62.449 | AX-94933305 | 7.175 | AX-94603548 | 7.535 | AX-95204866 | 0.597 | AX-94833113 | 11.72 | AX-94392788 | 9.138 |
| AX-94523644 | 8.923 | AX-95025260 | 62.449 | AX-94462783 | 7.175 | AX-94598750 | 7.535 | AX-94461272 | 0.597 | AX-94576868 | 11.72 | AX-94496296 | 9.138 |
| AX-95249630 | 8.923 | AX-94820297 | 62.449 | AX-95117759 | 7.175 | AX-95106345 | 7.535 | AX-94572080 | 0.597 | AX-94806979 | 12.12 | AX-95211609 | 9.138 |
| AX-94799150 | 8.923 | AX-94848912 | 67.017 | AX-94557337 | 7.175 | AX-94622376 | 7.535 | AX-94581018 | 0.597 | AX-94749505 | 12.12 | AX-94440399 | 9.138 |
| AX-94757643 | 9.925 | AX-94927151 | 67.017 | AX-94852069 | 7.175 | AX-95204902 | 7.535 | AX-94712402 | 0.597 | AX-94892032 | 12.12 | AX-94592672 | 9.138 |
| AX-95135434 | 9.925 | AX-95000163 | 67.216 | AX-94432825 | 7.175 | AX-94757012 | 7.535 | AX-95016611 | 0.597 | AX-94864700 | 12.32 | AX-95025999 | 9.138 |

|             |       |             |        |             |       |             |       |             |       |             |       |             |        |
|-------------|-------|-------------|--------|-------------|-------|-------------|-------|-------------|-------|-------------|-------|-------------|--------|
| AX-94850114 | 9.925 | AX-94996041 | 67.216 | AX-95168786 | 7.175 | AX-95094283 | 7.535 | AX-94772679 | 0.597 | AX-95141129 | 12.72 | AX-94787077 | 10.343 |
| AX-94539822 | 9.925 | AX-94804621 | 67.216 | AX-94537117 | 7.175 | AX-94388894 | 7.535 | AX-94557209 | 0.597 | AX-95198965 | 13.93 | AX-95098801 | 10.343 |
| AX-94802682 | 9.925 | AX-94442882 | 67.216 | AX-94828085 | 7.175 | AX-94647347 | 7.535 | AX-94438521 | 0.597 | AX-94424522 | 16.57 | AX-94513417 | 10.542 |
| AX-94700304 | 9.925 | AX-94573161 | 67.216 | AX-94758785 | 7.175 | AX-94532606 | 7.535 | AX-94537388 | 0.597 | AX-94504311 | 16.57 | AX-94461995 | 10.542 |
| AX-94683105 | 9.925 | AX-94398482 | 67.216 | AX-94635456 | 7.175 | AX-94504379 | 7.535 | AX-94943750 | 0.597 | AX-94850564 | 17.98 | AX-94703386 | 10.542 |
| AX-94980220 | 9.925 | AX-95188544 | 67.216 | AX-94650081 | 7.175 | AX-94998878 | 7.535 | AX-94449783 | 0.597 | AX-94449353 | 17.98 | AX-95140865 | 10.542 |
| AX-95248934 | 9.925 | AX-94636247 | 67.216 | AX-94420283 | 7.374 | AX-94427859 | 7.535 | AX-94474447 | 0.597 | AX-94768578 | 17.98 | AX-94477009 | 10.542 |
| AX-94646909 | 9.925 | AX-94799451 | 67.216 | AX-94481701 | 7.374 | AX-94759063 | 7.535 | AX-95209083 | 0.597 | AX-94605694 | 17.98 | AX-94468508 | 10.542 |
| AX-95116095 | 9.925 | AX-95257444 | 67.216 | AX-95236639 | 7.374 | AX-94956375 | 7.535 | AX-95203559 | 0.597 | AX-95075166 | 17.98 | AX-95012932 | 10.542 |
| AX-94683891 | 9.925 | AX-94643588 | 67.415 | AX-94878149 | 7.374 | AX-95247827 | 7.535 | AX-95005781 | 0.597 | AX-94985586 | 17.98 | AX-94757080 | 10.542 |

|             |       |             |        |             |       |             |       |             |       |             |       |             |        |
|-------------|-------|-------------|--------|-------------|-------|-------------|-------|-------------|-------|-------------|-------|-------------|--------|
| AX-94561589 | 9.925 | AX-95102088 | 67.415 | AX-95179710 | 7.374 | AX-94494882 | 7.535 | AX-95078475 | 0.597 | AX-94833031 | 17.98 | AX-95185374 | 10.542 |
| AX-94585755 | 9.925 | AX-95145587 | 75.354 | AX-94650068 | 7.374 | AX-94737285 | 7.535 | AX-95233050 | 0.597 | AX-95204328 | 17.98 | AX-95069098 | 10.542 |
| AX-95192433 | 9.925 | AX-94543423 | 75.354 | AX-95076840 | 7.374 | AX-95124640 | 7.535 | AX-94547737 | 0.597 | AX-94815009 | 17.98 | AX-95087314 | 10.542 |
| AX-94869141 | 9.925 | AX-95248938 | 75.354 | AX-94643635 | 7.374 | AX-95232031 | 7.535 | AX-94695126 | 0.597 | AX-94537752 | 17.98 | AX-94813599 | 10.542 |
| AX-94430783 | 9.925 | AX-94922568 | 75.354 | AX-94509895 | 7.374 | AX-94609301 | 7.535 | AX-94562455 | 0.597 | AX-94395800 | 17.98 | AX-94642678 | 10.542 |
| AX-94422013 | 9.925 | AX-95244410 | 75.354 | AX-94595143 | 7.374 | AX-94381472 | 7.535 | AX-94995549 | 0.597 | AX-94857959 | 17.98 | AX-94591682 | 10.542 |
| AX-94773154 | 9.925 | AX-94520053 | 75.354 | AX-94820446 | 7.374 | AX-95078354 | 7.535 | AX-94576796 | 0.597 | AX-94520215 | 17.98 | AX-95022091 | 10.542 |
| AX-94614192 | 9.925 | AX-94565835 | 75.354 | AX-95120296 | 7.374 | AX-94384455 | 7.535 | AX-94954394 | 0.597 | AX-95187810 | 17.98 | AX-94722175 | 10.542 |
| AX-94503286 | 9.925 | AX-94622361 | 75.354 | AX-94598785 | 7.374 | AX-94752140 | 7.535 | AX-94727515 | 0.597 | AX-94554856 | 17.98 | AX-95171374 | 10.542 |
| AX-94612467 | 9.925 | AX-94729799 | 75.354 | AX-94848436 | 7.374 | AX-94693037 | 7.535 | AX-94686719 | 0.597 | AX-94505868 | 17.98 | AX-94573994 | 10.542 |



|             |       |             |        |             |       |             |       |             |       |             |       |             |        |
|-------------|-------|-------------|--------|-------------|-------|-------------|-------|-------------|-------|-------------|-------|-------------|--------|
| AX-94956395 | 9.925 | AX-94545989 | 75.354 | AX-94487316 | 7.374 | AX-95175309 | 7.535 | AX-94944074 | 0.597 | AX-95099607 | 17.98 | AX-94887274 | 10.542 |
| AX-94670790 | 9.925 | AX-95103321 | 75.354 | AX-94748734 | 7.374 | AX-94755819 | 7.535 | AX-94713628 | 0.597 | AX-94952781 | 17.98 | AX-94439797 | 10.542 |
| AX-95202310 | 9.925 | AX-95107419 | 75.354 | AX-94876768 | 7.374 | AX-94830400 | 7.535 | AX-94949904 | 0.597 | AX-95228794 | 17.98 | AX-95249077 | 10.542 |
| AX-94675811 | 9.925 | AX-94923150 | 75.354 | AX-94725228 | 7.374 | AX-95242684 | 7.535 | AX-94928627 | 0.597 | AX-94989784 | 17.98 | AX-94951234 | 10.542 |
| AX-94621583 | 9.925 | AX-94662232 | 75.553 | AX-95075934 | 7.374 | AX-95131608 | 7.535 | AX-94568793 | 0.597 | AX-94529477 | 17.98 | AX-94870776 | 10.542 |
| AX-95014105 | 9.925 | AX-94496446 | 75.553 | AX-94824180 | 7.374 | AX-94885080 | 7.535 | AX-95173607 | 0.597 | AX-95096573 | 17.98 | AX-94944928 | 10.542 |
| AX-94390546 | 9.925 | AX-94675290 | 75.553 | AX-94557285 | 7.374 | AX-94584608 | 7.535 | AX-94830255 | 0.597 | AX-94542050 | 17.98 | AX-94729128 | 10.542 |
| AX-95255108 | 9.925 | AX-94866432 | 75.752 | AX-95250462 | 7.374 | AX-94530066 | 7.535 | AX-94795467 | 0.597 | AX-94388290 | 18.58 | AX-94826187 | 10.741 |
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| AX-94614812 | 9.925 | AX-94465410 | 75.752 | AX-94759455 | 7.374 | AX-94393357 | 9.354 | AX-94456888 | 0.597 | AX-94535645 | 18.58 | AX-94533947 | 10.741 |

|             |       |             |        |             |       |             |       |             |       |             |       |             |        |
|-------------|-------|-------------|--------|-------------|-------|-------------|-------|-------------|-------|-------------|-------|-------------|--------|
| AX-94592111 | 9.925 | AX-94491641 | 75.752 | AX-94529019 | 7.374 | AX-95241651 | 9.354 | AX-95201458 | 0.597 | AX-94790040 | 18.58 | AX-95025990 | 10.741 |
| AX-94606585 | 9.925 | AX-94426164 | 76.15  | AX-94609310 | 7.374 | AX-94739374 | 9.354 | AX-94540905 | 0.597 | AX-94930552 | 18.58 | AX-94709622 | 10.94  |
| AX-94509407 | 9.925 | AX-94717863 | 76.15  | AX-94530970 | 7.374 | AX-94683511 | 9.552 | AX-94731226 | 0.597 | AX-94418078 | 18.58 | AX-94917299 | 10.94  |
| AX-94598167 | 9.925 | AX-94829763 | 76.15  | AX-94532509 | 7.374 | AX-94438118 | 12.62 | AX-94639902 | 0.597 | AX-94526165 | 18.58 | AX-94442538 | 10.94  |
| AX-94658526 | 9.925 | AX-94401625 | 76.15  | AX-94667742 | 7.374 | AX-95250431 | 12.62 | AX-94848929 | 0.597 | AX-95180847 | 18.58 | AX-94629085 | 10.94  |
| AX-94879002 | 9.925 | AX-94680070 | 76.15  | AX-95194831 | 7.374 | AX-94744874 | 12.62 | AX-94873783 | 0.597 | AX-94596423 | 18.58 | AX-94692932 | 11.138 |
| AX-94518543 | 9.925 | AX-94811232 | 76.15  | AX-95094335 | 7.374 | AX-94620312 | 12.62 | AX-95123476 | 0.597 | AX-94946420 | 18.58 | AX-94409225 | 11.138 |
| AX-94602515 | 9.925 | AX-95166376 | 76.15  | AX-95127800 | 7.374 | AX-94531979 | 12.62 | AX-94650952 | 0.597 | AX-94721919 | 18.58 | AX-95195229 | 11.138 |
| AX-94809557 | 9.925 | AX-94633053 | 76.15  | AX-95182852 | 7.374 | AX-95181779 | 13.42 | AX-94545064 | 0.597 | AX-95249670 | 18.58 | AX-94980258 | 11.138 |
| AX-95248891 | 9.925 | AX-94944759 | 76.15  | AX-94723317 | 7.374 | AX-94881260 | 15.65 | AX-95191670 | 0.597 | AX-94904341 | 18.58 | AX-94795562 | 11.138 |

|             |       |             |        |             |       |             |       |             |       |             |       |             |        |
|-------------|-------|-------------|--------|-------------|-------|-------------|-------|-------------|-------|-------------|-------|-------------|--------|
| AX-95231223 | 9.925 | AX-94697054 | 76.15  | AX-94422471 | 7.374 | AX-95101430 | 15.65 | AX-95138537 | 0.597 | AX-94634321 | 18.58 | AX-94464494 | 11.138 |
| AX-94692179 | 9.925 | AX-94435610 | 76.15  | AX-94781891 | 7.374 | AX-95136476 | 15.65 | AX-95104768 | 0.597 | AX-94558532 | 18.58 | AX-94464859 | 11.938 |
| AX-95243490 | 9.925 | AX-94583058 | 76.15  | AX-94561850 | 7.374 | AX-94776666 | 15.65 | AX-95180651 | 0.597 | AX-94593655 | 18.58 | AX-94804415 | 11.938 |
| AX-94959080 | 9.925 | AX-95201233 | 76.15  | AX-94458575 | 7.374 | AX-94561861 | 15.65 | AX-95137982 | 0.597 | AX-94617550 | 18.58 | AX-94506247 | 11.938 |
| AX-94459935 | 9.925 | AX-94571673 | 76.15  | AX-94733983 | 7.374 | AX-94751291 | 15.65 | AX-94533870 | 0.597 | AX-94729031 | 18.58 | AX-94832691 | 11.938 |
| AX-95215437 | 9.925 | AX-95073495 | 77.559 | AX-94491139 | 7.374 | AX-94437486 | 15.65 | AX-94410375 | 0.597 | AX-95213399 | 18.58 | AX-94599966 | 11.938 |
| AX-94498159 | 9.925 | AX-94384755 | 77.559 | AX-94585873 | 7.374 | AX-94700104 | 17.06 | AX-94838552 | 0.597 | AX-94950771 | 18.58 | AX-94494567 | 11.938 |
| AX-94481203 | 9.925 | AX-95095062 | 77.559 | AX-95000311 | 7.374 | AX-94651716 | 17.06 | AX-94991733 | 0.597 | AX-94927291 | 18.58 | AX-94527347 | 11.938 |
| AX-94962111 | 9.925 | AX-94678354 | 77.559 | AX-95113387 | 7.374 | AX-94790289 | 17.06 | AX-94717880 | 0.597 | AX-95196531 | 18.58 | AX-95197082 | 11.938 |
| AX-95114845 | 9.925 | AX-94783812 | 77.559 | AX-94478227 | 7.374 | AX-95206487 | 17.06 | AX-94847038 | 0.597 | AX-94735016 | 18.58 | AX-94759866 | 11.938 |

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|-------------|--------|-------------|--------|-------------|-------|-------------|-------|-------------|-------|-------------|-------|-------------|--------|
| AX-95126278 | 9.925  | AX-94886208 | 77.559 | AX-94492488 | 7.374 | AX-94653077 | 17.06 | AX-95255472 | 0.597 | AX-94728133 | 18.58 | AX-94702512 | 11.938 |
| AX-94911307 | 9.925  | AX-94797457 | 77.559 | AX-94393636 | 7.374 | AX-94705196 | 17.06 | AX-94932238 | 0.597 | AX-94508661 | 18.58 | AX-94413444 | 11.938 |
| AX-95161096 | 12.365 | AX-95143980 | 77.559 | AX-94701303 | 7.374 | AX-94515235 | 17.06 | AX-94556216 | 0.597 | AX-94803509 | 18.58 | AX-94428847 | 11.938 |
| AX-94406596 | 12.763 | AX-95101757 | 77.559 | AX-95147238 | 7.374 | AX-94568397 | 17.46 | AX-94928650 | 0.597 | AX-94494541 | 18.58 | AX-95078053 | 11.938 |
| AX-94521730 | 12.763 | AX-95235860 | 77.559 | AX-94707410 | 7.772 | AX-95078142 | 19.48 | AX-95017218 | 0.597 | AX-94915188 | 18.58 | AX-94415990 | 11.938 |
| AX-94653440 | 12.763 | AX-95236664 | 77.559 | AX-94766553 | 7.772 | AX-94394417 | 21.51 | AX-95248802 | 0.597 | AX-94384949 | 18.58 | AX-95183704 | 11.938 |
| AX-95146900 | 13.162 | AX-95160157 | 77.559 | AX-94458177 | 7.772 | AX-94699138 | 21.51 | AX-94978617 | 0.597 | AX-95213663 | 18.58 | AX-94899308 | 11.938 |
| AX-94957945 | 13.162 | AX-94462033 | 77.559 | AX-94652746 | 7.772 | AX-94593455 | 21.51 | AX-95256409 | 0.597 | AX-95112779 | 18.58 | AX-94826977 | 11.938 |
| AX-94962619 | 13.162 | AX-94545814 | 77.559 | AX-95116911 | 8.371 | AX-95215789 | 21.51 | AX-95190014 | 0.597 | AX-94500606 | 18.58 | AX-94649688 | 11.938 |
| AX-95101674 | 13.162 | AX-94905415 | 77.559 | AX-94874294 | 8.371 | AX-94389401 | 21.51 | AX-95080399 | 0.597 | AX-94524890 | 18.58 | AX-94430614 | 11.938 |

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| AX-94936271 | 13.162 | AX-94531805 | 77.559 | AX-94786270 | 8.371 | AX-94818645 | 21.51 | AX-94768745 | 0.597 | AX-94568697 | 18.58 | AX-94858964 | 11.938 |
| AX-95138787 | 13.162 | AX-94948817 | 77.559 | AX-95013828 | 8.371 | AX-94806523 | 21.51 | AX-94539430 | 0.597 | AX-94807105 | 18.58 | AX-95239412 | 11.938 |
| AX-94482529 | 13.162 | AX-94432299 | 77.559 | AX-95071605 | 8.371 | AX-94881881 | 21.71 | AX-95122485 | 0.597 | AX-94562108 | 18.58 | AX-95235263 | 11.938 |
| AX-94539098 | 13.162 | AX-95070414 | 77.559 | AX-94619859 | 8.371 | AX-94570846 | 21.71 | AX-94489313 | 0.597 | AX-94837802 | 18.58 | AX-95127764 | 11.938 |
| AX-94674334 | 13.162 | AX-94867768 | 77.559 | AX-94686804 | 8.371 | AX-95078506 | 21.91 | AX-94876980 | 0.597 | AX-94825170 | 18.58 | AX-95124586 | 11.938 |
| AX-95149143 | 13.162 | AX-94815377 | 77.559 | AX-95171624 | 8.371 | AX-95087500 | 21.91 | AX-94525198 | 0.597 | AX-94713921 | 18.58 | AX-94652768 | 11.938 |
| AX-94425479 | 13.162 | AX-95091800 | 77.559 | AX-94765040 | 8.371 | AX-95184640 | 21.91 | AX-95237695 | 0.597 | AX-94550826 | 18.58 | AX-94511634 | 11.938 |
| AX-95196261 | 13.162 | AX-94429561 | 77.559 | AX-94741448 | 8.371 | AX-94436194 | 21.91 | AX-94498254 | 0.597 | AX-94985428 | 18.58 | AX-95198132 | 11.938 |
| AX-94449858 | 13.162 | AX-94490752 | 77.559 | AX-95629843 | 8.371 | AX-95176204 | 21.91 | AX-95208153 | 0.597 | AX-94805155 | 18.58 | AX-94998671 | 11.938 |
| AX-95260090 | 13.162 | AX-94522936 | 77.559 | AX-94926538 | 8.371 | AX-95095804 | 21.91 | AX-94717296 | 0.597 | AX-94416966 | 18.58 | AX-94837838 | 11.938 |

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| AX-94914133 | 13.162 | AX-94897254 | 77.559 | AX-94693579 | 8.371 | AX-94524286 | 21.91 | AX-94922739 | 0.597 | AX-94539511 | 18.58 | AX-94951115 | 11.938 |
| AX-95163352 | 13.162 | AX-94573713 | 77.559 | AX-94936012 | 8.371 | AX-94510113 | 21.91 | AX-95105528 | 0.597 | AX-94575930 | 18.58 | AX-94837175 | 13.347 |
| AX-94784918 | 13.162 | AX-94980871 | 77.559 | AX-94737201 | 8.371 | AX-94895095 | 22.11 | AX-94475918 | 0.597 | AX-94541273 | 18.58 | AX-94838069 | 13.347 |
| AX-94648380 | 13.162 | AX-94522494 | 77.559 | AX-95167675 | 8.371 | AX-94782348 | 22.11 | AX-94536128 | 0.597 | AX-94405863 | 18.58 | AX-94704090 | 13.347 |
| AX-94425295 | 13.162 | AX-86176645 | 77.758 | AX-94710473 | 8.371 | AX-94669430 | 22.11 | AX-94407767 | 0.597 | AX-95171109 | 18.58 | AX-94526306 | 13.347 |
| AX-95074428 | 13.162 | AX-95086310 | 77.758 | AX-94878840 | 8.371 | AX-95008348 | 22.11 | AX-94409635 | 0.597 | AX-94802955 | 18.58 | AX-94383838 | 13.347 |
| AX-94407295 | 13.162 | AX-94494284 | 79.576 | AX-94742844 | 8.371 | AX-95160280 | 22.11 | AX-94783912 | 0.597 | AX-95118160 | 18.58 | AX-95240436 | 13.347 |
| AX-94929459 | 13.162 | AX-94386926 | 79.576 | AX-95022568 | 8.371 | AX-94809047 | 22.11 | AX-94483563 | 0.597 | AX-95189674 | 18.58 | AX-94734620 | 13.347 |
| AX-95096429 | 13.162 | AX-95192669 | 79.576 | AX-94551787 | 8.371 | AX-94854013 | 22.11 | AX-94445027 | 0.597 | AX-94400016 | 18.58 | AX-94560586 | 13.347 |
| AX-94739534 | 13.162 | AX-95013279 | 79.576 | AX-94395570 | 8.371 | AX-94429994 | 22.11 | AX-95206034 | 0.597 | AX-94553420 | 18.58 | AX-94525211 | 13.347 |

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|-------------|--------|-------------|--------|-------------|-------|-------------|-------|-------------|-------|-------------|-------|-------------|--------|
| AX-94472834 | 13.162 | AX-94875269 | 79.576 | AX-94588325 | 8.371 | AX-94938754 | 22.11 | AX-94632506 | 0.597 | AX-94437050 | 18.58 | AX-94406645 | 13.347 |
| AX-94572667 | 13.162 | AX-94662597 | 79.576 | AX-94617587 | 8.371 | AX-94494307 | 22.11 | AX-94985905 | 0.597 | AX-94664691 | 18.78 | AX-95226247 | 13.347 |
| AX-94391230 | 13.162 | AX-94474518 | 79.576 | AX-95241340 | 8.371 | AX-94452650 | 22.11 | AX-94773977 | 0.597 | AX-94479603 | 18.78 | AX-95105700 | 13.347 |
| AX-94463471 | 13.162 | AX-95119484 | 79.576 | AX-94495018 | 8.371 | AX-94830126 | 22.11 | AX-94571981 | 0.597 | AX-94633473 | 18.78 | AX-94419014 | 13.347 |
| AX-94976593 | 13.162 | AX-94790257 | 79.775 | AX-95102903 | 8.371 | AX-94448481 | 22.11 | AX-94509671 | 0.597 | AX-94964128 | 18.78 | AX-94443167 | 13.347 |
| AX-94649087 | 13.162 | AX-94636073 | 79.974 | AX-94392142 | 8.371 | AX-94638862 | 22.11 | AX-94424457 | 0.597 | AX-94640991 | 18.78 | AX-95200253 | 13.347 |
| AX-94595844 | 13.162 | AX-94768754 | 90.051 | AX-95114368 | 8.371 | AX-94419987 | 22.11 | AX-94999407 | 0.597 | AX-94509964 | 18.78 | AX-94508582 | 13.546 |
| AX-94846017 | 13.162 | AX-94982210 | 90.25  | AX-95002760 | 8.371 | AX-94418873 | 22.11 | AX-94595799 | 0.597 | AX-95112325 | 18.78 | AX-94538568 | 13.546 |
| AX-94548150 | 13.162 | AX-95087762 | 90.25  | AX-94759675 | 8.371 | AX-95179840 | 22.11 | AX-94420960 | 0.597 | AX-94495972 | 18.78 | AX-94468312 | 13.546 |
| AX-95131413 | 13.162 | AX-94602367 | 90.25  | AX-94840232 | 8.371 | AX-94950648 | 22.11 | AX-94624188 | 5.165 | AX-94778763 | 18.78 | AX-94492797 | 13.546 |

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| AX-94563128 | 13.162 | AX-94410030 | 90.25  | AX-94681165 | 8.371 | AX-94418120 | 22.11 | AX-94932813 | 6.778 | AX-94734825 | 18.78 | AX-95187839 | 13.745 |
| AX-94811680 | 13.162 | AX-94802872 | 90.25  | AX-94672108 | 8.371 | AX-94510143 | 22.11 | AX-94830054 | 6.778 | AX-94478906 | 18.78 | AX-94532247 | 13.745 |
| AX-94400504 | 13.162 | AX-94382232 | 90.25  | AX-95115219 | 8.371 | AX-95178063 | 22.11 | AX-95175236 | 6.778 | AX-95168728 | 18.78 | AX-94902683 | 15.769 |
| AX-95222741 | 13.162 | AX-94557592 | 90.25  | AX-94523038 | 8.371 | AX-94788550 | 22.91 | AX-94608425 | 6.778 | AX-94653761 | 18.78 | AX-94440464 | 15.769 |
| AX-94615574 | 13.162 | AX-95126627 | 92.275 | AX-94412171 | 8.57  |             |       | AX-94619669 | 6.778 | AX-94575371 | 18.78 | AX-94501715 | 15.769 |
| AX-94524256 | 13.162 | AX-94390156 | 92.275 | AX-94743899 | 8.57  |             |       | AX-94468234 | 6.778 | AX-94763348 | 18.78 | AX-94718151 | 15.769 |
| AX-94400160 | 13.162 | AX-94746152 | 92.275 | AX-94691425 | 8.57  |             |       | AX-94515428 | 6.778 | AX-94655232 | 18.78 | AX-95168005 | 15.769 |
| AX-95653055 | 13.162 | AX-94587803 | 92.275 | AX-94655585 | 8.769 |             |       | AX-95244062 | 10.7  | AX-94915758 | 18.78 | AX-94944057 | 15.769 |
| AX-94416573 | 13.162 | AX-94937792 | 95.132 | AX-94961316 | 8.769 |             |       | AX-95092667 | 11.1  | AX-94789329 | 18.78 | AX-94451716 | 15.769 |
| AX-95021590 | 13.162 | AX-94570110 | 97.364 | AX-94470995 | 8.769 |             |       | AX-94541972 | 11.1  | AX-95120253 | 19.18 | AX-95154545 | 15.769 |



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|-------------|--------|-------------|--------|-------------|-------|--|--|-------------|-------|-------------|-------|-------------|--------|
| AX-95021598 | 13.162 | AX-95180614 | 97.364 | AX-94519442 | 8.769 |  |  | AX-94585720 | 11.29 | AX-95141417 | 19.18 | AX-94757715 | 15.769 |
| AX-94995843 | 13.162 | AX-95106164 | 97.364 | AX-94431474 | 8.968 |  |  | AX-95181941 | 11.29 | AX-94619243 | 19.18 | AX-94721448 | 15.769 |
| AX-94461345 | 13.162 | AX-95123223 | 106.01 | AX-94933078 | 9.166 |  |  | AX-94924130 | 11.29 | AX-94547577 | 19.18 | AX-94889553 | 15.769 |
| AX-95232405 | 13.162 | AX-94413948 | 106.41 | AX-95099187 | 9.365 |  |  | AX-94641760 | 11.29 | AX-94699632 | 19.18 | AX-94617441 | 15.769 |
| AX-95217273 | 13.162 | AX-94631566 | 106.41 |             |       |  |  | AX-94650050 | 11.29 | AX-94498894 | 19.18 | AX-94513629 | 15.769 |
| AX-95115653 | 13.162 | AX-95220774 | 109.9  |             |       |  |  | AX-94656240 | 11.29 | AX-95082377 | 19.18 | AX-95179721 | 15.769 |
| AX-94577493 | 13.162 | AX-94430439 | 109.9  |             |       |  |  | AX-94781016 | 11.89 | AX-94613684 | 19.18 | AX-94679028 | 15.769 |
| AX-94575557 | 13.162 | AX-95141462 | 109.9  |             |       |  |  | AX-94718281 | 12.9  | AX-94466211 | 19.18 | AX-95081313 | 15.769 |
| AX-94577061 | 13.162 | AX-95090223 | 110.5  |             |       |  |  | AX-94471407 | 12.9  | AX-94618570 | 19.18 | AX-94987166 | 15.769 |
| AX-95145603 | 13.162 | AX-94603061 | 110.89 |             |       |  |  | AX-94844464 | 13.09 | AX-94925103 | 19.18 | AX-95138960 | 15.769 |

|             |        |             |        |  |  |  |  |             |       |             |       |             |        |
|-------------|--------|-------------|--------|--|--|--|--|-------------|-------|-------------|-------|-------------|--------|
| AX-94861819 | 13.162 | AX-95630025 | 111.09 |  |  |  |  | AX-95137963 | 13.09 | AX-95165090 | 19.18 | AX-94553589 | 17.794 |
| AX-94434085 | 13.962 | AX-86165433 | 111.09 |  |  |  |  | AX-94392411 | 13.09 | AX-94590270 | 19.18 | AX-94728054 | 17.794 |
| AX-95134864 | 13.962 | AX-94841174 | 130.77 |  |  |  |  | AX-94628803 | 13.09 | AX-94986070 | 19.18 | AX-95198644 | 17.794 |
| AX-94764959 | 13.962 | AX-95182708 | 138.48 |  |  |  |  | AX-94461707 | 13.29 | AX-94594704 | 19.18 | AX-94824785 | 18.999 |
| AX-95002730 | 13.962 | AX-95221446 | 138.48 |  |  |  |  | AX-94456002 | 13.29 | AX-94598181 | 19.18 | AX-94923533 | 18.999 |
| AX-94797466 | 13.962 | AX-94410588 | 188.32 |  |  |  |  | AX-94866362 | 13.29 | AX-94532490 | 19.38 | AX-94476499 | 18.999 |
| AX-95232857 | 13.962 | AX-94479933 | 188.32 |  |  |  |  | AX-95240372 | 13.29 | AX-94425038 | 20.79 | AX-94701057 | 18.999 |
| AX-94795738 | 13.962 |             |        |  |  |  |  | AX-94810321 | 13.29 | AX-94943884 | 20.79 | AX-94847772 | 18.999 |
| AX-95228723 | 13.962 |             |        |  |  |  |  | AX-94532204 | 13.29 | AX-95246447 | 20.79 | AX-95191553 | 18.999 |
| AX-95208655 | 13.962 |             |        |  |  |  |  | AX-94671630 | 13.29 | AX-94661844 | 22.19 | AX-94639557 | 18.999 |

|             |        |  |  |  |  |  |  |             |       |             |       |             |        |
|-------------|--------|--|--|--|--|--|--|-------------|-------|-------------|-------|-------------|--------|
| AX-94416954 | 13.962 |  |  |  |  |  |  | AX-94412685 | 13.49 | AX-94907600 | 22.19 | AX-94479709 | 18.999 |
| AX-94693688 | 13.962 |  |  |  |  |  |  | AX-95194904 | 20.06 | AX-94439560 | 22.19 | AX-95121088 | 18.999 |
| AX-95091170 | 16.193 |  |  |  |  |  |  | AX-95007739 | 20.25 | AX-94827219 | 22.19 | AX-94469418 | 18.999 |
| AX-94813554 | 16.193 |  |  |  |  |  |  | AX-95117413 | 21.05 | AX-95008276 | 22.19 | AX-94414073 | 18.999 |
| AX-94419566 | 16.993 |  |  |  |  |  |  | AX-94983233 | 21.05 | AX-95231080 | 22.19 | AX-94698891 | 18.999 |
| AX-94527933 | 19.225 |  |  |  |  |  |  | AX-94428806 | 21.05 | AX-94635598 | 22.19 | AX-94854917 | 18.999 |
|             |        |  |  |  |  |  |  | AX-94724199 | 21.05 | AX-94761123 | 22.19 | AX-94399369 | 18.999 |
|             |        |  |  |  |  |  |  | AX-94399413 | 21.05 | AX-94467190 | 22.19 | AX-95203693 | 18.999 |
|             |        |  |  |  |  |  |  | AX-94748768 | 21.05 | AX-94468535 | 22.39 | AX-94778935 | 18.999 |
|             |        |  |  |  |  |  |  | AX-89745601 | 21.25 | AX-94572840 | 23.8  | AX-95200001 | 22.918 |

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|--|--|--|--|--|--|--|--|-----------------|-------|-----------------|-------|-----------------|--------|
|  |  |  |  |  |  |  |  | AX-<br>94573573 | 24.32 | AX-<br>94691016 | 23.8  | AX-<br>94422289 | 22.918 |
|  |  |  |  |  |  |  |  | AX-<br>94915329 | 27.39 | AX-<br>95015449 | 23.8  | AX-<br>94621214 | 22.918 |
|  |  |  |  |  |  |  |  | AX-<br>94749233 | 27.39 | AX-<br>94864744 | 23.8  |                 |        |
|  |  |  |  |  |  |  |  | AX-<br>94691860 | 27.39 | AX-<br>94697250 | 25.83 |                 |        |
|  |  |  |  |  |  |  |  | AX-<br>95242385 | 27.39 | AX-<br>95223897 | 26.02 |                 |        |
|  |  |  |  |  |  |  |  | AX-<br>94637076 | 27.39 | AX-<br>94558017 | 26.02 |                 |        |
|  |  |  |  |  |  |  |  | AX-<br>94897713 | 27.39 | AX-<br>94757279 | 31.47 |                 |        |
|  |  |  |  |  |  |  |  | AX-<br>95107195 | 27.59 | AX-<br>94585049 | 33.08 |                 |        |
|  |  |  |  |  |  |  |  | AX-<br>86177094 | 27.79 | AX-<br>94599180 | 33.68 |                 |        |
|  |  |  |  |  |  |  |  | AX-<br>94737123 | 27.79 | AX-<br>94404191 | 39.13 |                 |        |

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|--|--|--|--|--|--|--|--|-----------------|-------|-----------------|-------|--|--|
|  |  |  |  |  |  |  |  | AX-<br>95209690 | 27.79 | AX-<br>94930314 | 39.13 |  |  |
|  |  |  |  |  |  |  |  | AX-<br>94790093 | 27.79 | AX-<br>94759235 | 40.33 |  |  |
|  |  |  |  |  |  |  |  | AX-<br>94501718 | 27.79 | AX-<br>94681410 | 40.53 |  |  |
|  |  |  |  |  |  |  |  | AX-<br>94473466 | 27.79 | AX-<br>94706801 | 40.53 |  |  |
|  |  |  |  |  |  |  |  | AX-<br>95132630 | 27.79 | AX-<br>94844733 | 40.53 |  |  |
|  |  |  |  |  |  |  |  | AX-<br>94829193 | 27.79 | AX-<br>94676023 | 41.13 |  |  |
|  |  |  |  |  |  |  |  | AX-<br>95157769 | 27.79 | AX-<br>95249383 | 44.62 |  |  |
|  |  |  |  |  |  |  |  | AX-<br>94893504 | 27.79 |                 |       |  |  |
|  |  |  |  |  |  |  |  | AX-<br>95080756 | 27.99 |                 |       |  |  |
|  |  |  |  |  |  |  |  | AX-<br>94926022 | 29.4  |                 |       |  |  |

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|  |  |  |  |  |  |  |  | AX-<br>94633885 | 29.4  |  |  |  |  |
|  |  |  |  |  |  |  |  | AX-<br>94727436 | 29.4  |  |  |  |  |
|  |  |  |  |  |  |  |  | AX-<br>94831861 | 29.4  |  |  |  |  |
|  |  |  |  |  |  |  |  | AX-<br>94651710 | 29.99 |  |  |  |  |
|  |  |  |  |  |  |  |  | AX-<br>95162146 | 30.19 |  |  |  |  |
|  |  |  |  |  |  |  |  | AX-<br>94401947 | 30.19 |  |  |  |  |
|  |  |  |  |  |  |  |  | AX-<br>95182970 | 30.19 |  |  |  |  |
|  |  |  |  |  |  |  |  | AX-<br>94546890 | 31.4  |  |  |  |  |
|  |  |  |  |  |  |  |  | AX-<br>94491624 | 31.4  |  |  |  |  |
|  |  |  |  |  |  |  |  | AX-<br>94672688 | 31.4  |  |  |  |  |

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|  |  |  |  |  |  |  |  | AX-<br>94764219 | 31.4  |  |  |  |  |
|  |  |  |  |  |  |  |  | AX-<br>94925161 | 31.4  |  |  |  |  |
|  |  |  |  |  |  |  |  | AX-<br>94622408 | 31.8  |  |  |  |  |
|  |  |  |  |  |  |  |  | AX-<br>95177900 | 31.8  |  |  |  |  |
|  |  |  |  |  |  |  |  | AX-<br>94732135 | 31.8  |  |  |  |  |
|  |  |  |  |  |  |  |  | AX-<br>94960260 | 31.99 |  |  |  |  |
|  |  |  |  |  |  |  |  | AX-<br>94571628 | 31.99 |  |  |  |  |
|  |  |  |  |  |  |  |  | AX-<br>95215143 | 31.99 |  |  |  |  |
|  |  |  |  |  |  |  |  | AX-<br>95126208 | 36.13 |  |  |  |  |
|  |  |  |  |  |  |  |  | AX-<br>95232272 | 36.13 |  |  |  |  |

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|  |  |  |  |  |  |  |  | AX-<br>94401980 | 36.13 |  |  |  |  |
|  |  |  |  |  |  |  |  | AX-<br>95098994 | 36.13 |  |  |  |  |
|  |  |  |  |  |  |  |  | AX-<br>94624003 | 36.33 |  |  |  |  |
|  |  |  |  |  |  |  |  | AX-<br>94508002 | 36.33 |  |  |  |  |
|  |  |  |  |  |  |  |  | AX-<br>94626891 | 36.33 |  |  |  |  |
|  |  |  |  |  |  |  |  | AX-<br>94541369 | 37.33 |  |  |  |  |
|  |  |  |  |  |  |  |  | AX-<br>94705918 | 38.94 |  |  |  |  |
|  |  |  |  |  |  |  |  | AX-<br>94467792 | 39.34 |  |  |  |  |
|  |  |  |  |  |  |  |  | AX-<br>94997202 | 40.34 |  |  |  |  |
|  |  |  |  |  |  |  |  | AX-<br>94385363 | 40.54 |  |  |  |  |



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|  |  |  |  |  |  |  |  | AX-<br>94573720 | 40.74 |  |  |  |  |
|  |  |  |  |  |  |  |  | AX-<br>94839083 | 40.74 |  |  |  |  |
|  |  |  |  |  |  |  |  | AX-<br>94826616 | 40.74 |  |  |  |  |
|  |  |  |  |  |  |  |  | AX-<br>94572247 | 40.74 |  |  |  |  |
|  |  |  |  |  |  |  |  | AX-<br>94694080 | 40.94 |  |  |  |  |
|  |  |  |  |  |  |  |  | AX-<br>94659800 | 46.16 |  |  |  |  |
|  |  |  |  |  |  |  |  | AX-<br>95019550 | 46.16 |  |  |  |  |
|  |  |  |  |  |  |  |  | AX-<br>95091359 | 47.98 |  |  |  |  |
|  |  |  |  |  |  |  |  | AX-<br>94520836 | 48.18 |  |  |  |  |

## Appendix 4

Presence (+) and absence (-) of introgressions from linkage group of *T. timopheevii* in respective families used for genotyping (a family is the BC<sub>1</sub> and all of the later generations which generated from the same BC<sub>1</sub>).

| Families | Individuals | LG1            |   | LG2            |   | LG3            |   | LG4            |   | LG5            |   | LG6            |   | LG7            |   |    |
|----------|-------------|----------------|---|----------------|---|----------------|---|----------------|---|----------------|---|----------------|---|----------------|---|----|
|          |             | A <sup>t</sup> | G | A <sup>t</sup> | G | A <sup>t</sup> | G | A <sup>t</sup> | G | A <sup>t</sup> | G | A <sup>t</sup> | G | A <sup>t</sup> | G |    |
| Family 1 | BC1-113A    | +              | - | -              | + | -              | - | +              | + | +              | + | +              | + | +              | + | 10 |
|          | BC2-258A    | -              | - | -              | + | -              | - | +              | + | -              | + | +              | + | +              | + | 8  |
|          | BC2-258B    | +              | - | -              | + | -              | - | -              | - | +              | + | -              | + | +              | + | 7  |
|          | BC3-91A     | -              | - | -              | + | -              | - | -              | - | -              | + | -              | - | +              | + | 4  |
|          | BC3-92A     | +              | - | -              | + | -              | - | -              | - | -              | + | -              | + | +              | - | 5  |
|          | BC2-258C    | -              | - | -              | + | -              | - | +              | + | -              | + | +              | + | +              | - | 7  |
|          | BC3-419A    | -              | - | -              | + | -              | - | -              | - | -              | + | +              | + | +              | - | 5  |
|          | BC2-359A    | +              | - | -              | + | -              | - | -              | - | -              | - | +              | + | +              | - | 5  |
|          | BC1-113B    | +              | - | +              | + | +              | + | +              | + | +              | - | +              | + | +              | + | 12 |
| Family 2 | BC2-259A    | -              | - | +              | + | -              | + | -              | + | -              | - | -              | + | -              | - | 5  |
|          | BC3-93A     | -              | - | -              | + | -              | + | -              | + | -              | - | -              | - | -              | - | 3  |
|          | BC3-93B     | -              | - | +              | - | -              | + | -              | - | -              | - | -              | + | -              | - | 3  |
|          | BC3-93C     | -              | - | -              | + | -              | + | -              | - | -              | - | -              | + | -              | - | 3  |
|          | BC2-259B    | -              | - | +              | + | -              | - | -              | + | +              | - | +              | - | -              | + | 6  |
|          | BC3-368A    | -              | - | -              | + | -              | - | -              | - | -              | - | +              | - | -              | + | 3  |
|          | BC3-368B    | -              | - | +              | + | -              | - | -              | - | -              | - | +              | - | -              | + | 4  |
|          | BC3-368C    | -              | - | -              | + | -              | - | -              | + | +              | - | -              | - | -              | + | 4  |
|          | BC2-259C    | +              | - | +              | + | +              | + | +              | - | +              | - | -              | + | +              | + | 10 |
|          | BC3-420A    | +              | - | -              | + | +              | + | +              | - | +              | - | -              | + | +              | + | 9  |
|          | BC3-420B    | -              | - | +              | - | +              | - | -              | - | +              | - | -              | + | -              | - | 4  |
|          | BC3-422A    | -              | - | +              | + | -              | + | -              | - | +              | - | -              | - | +              | + | 6  |
|          | BC3-422B    | +              | - | +              | + | -              | - | +              | - | +              | - | -              | + | +              | + | 8  |

|          |            |   |   |   |   |   |   |   |   |   |   |   |   |   |   |    |
|----------|------------|---|---|---|---|---|---|---|---|---|---|---|---|---|---|----|
|          | BC2-360A   | + | - | + | + | + | - | + | + | + | - | - | + | + | - | 9  |
|          | BC2-360B   | + | - | + | + | + | + | - | - | - | - | + | + | + | - | 8  |
|          | BC3-367A   | + | - | - | + | + | - | - | - | - | - | - | - | + | - | 4  |
|          | BC3-367B   | + | - | + | + | + | - | - | - | - | - | + | + | + | - | 7  |
| Family 3 | BC1-113C   | + | - | + | + | + | - | + | - | + | + | + | + | + | + | 11 |
|          | BC2-260A   | + | - | + | + | - | - | - | - | + | + | - | - | + | + | 7  |
|          | BC3-423B   | - | - | + | + | - | - | - | - | - | + | - | - | + | + | 5  |
|          | BC3-423C   | - | - | - | + | - | - | - | - | + | + | - | - | - | + | 4  |
|          | BC3-423D   | - | - | + | + | - | - | - | - | - | + | - | - | - | + | 4  |
|          | BC3-423E   | - | - | - | - | - | - | - | - | + | - | - | - | - | - | 1  |
|          | BC3-423F   | + | - | + | + | - | - | - | - | - | - | - | - | - | - | 3  |
|          | BC3-423G   | - | - | - | + | - | - | - | - | + | - | - | - | + | + | 4  |
|          | BC3-423H   | + | - | + | + | - | - | - | - | + | + | - | - | + | + | 7  |
|          | BC2-260B   | + | - | - | + | - | - | - | - | + | + | - | + | + | + | 7  |
|          | BC3-94A    | + | - | - | + | - | - | - | - | - | + | - | + | - | + | 5  |
|          | BC4-131A   | - | - | - | + | - | - | - | - | - | + | - | - | - | - | 2  |
|          | BC4-131B   | + | - | - | + | - | - | - | - | - | - | - | - | - | - | 2  |
|          | BC4-131C   | + | - | - | + | - | - | - | - | - | + | - | - | - | + | 4  |
|          | BC4-131D   | - | - | - | + | - | - | - | - | - | - | - | + | - | + | 3  |
|          | BC3F1-138A | - | - | - | + | - | - | - | - | - | + | - | - | - | - | 2  |
|          | BC3F1-138B | + | - | - | + | - | - | - | - | - | - | - | + | - | - | 3  |
|          | BC3F1-138C | - | - | - | + | - | - | - | - | - | - | - | + | - | - | 2  |
|          | BC3F1-138D | + | - | - | + | - | - | - | - | - | + | - | + | - | - | 4  |
|          | BC3-94B    | + | - | - | - | - | - | - | - | + | + | - | + | + | - | 5  |
|          | BC3-94C    | - | - | - | + | - | - | - | - | + | - | - | + | + | - | 4  |
|          | BC4-132A   | - | - | - | + | - | - | - | - | + | - | - | - | + | - | 3  |
|          | BC4-132B   | - | - | - | - | - | - | - | - | - | - | - | - | + | - | 1  |
|          | BC4-132C   | - | - | - | + | - | - | - | - | - | - | - | - | + | - | 2  |
|          | BC4-132D   | - | - | - | - | - | - | - | - | - | - | - | + | - | - | 1  |

|          |            |   |   |   |   |   |   |   |   |   |   |   |   |   |   |    |
|----------|------------|---|---|---|---|---|---|---|---|---|---|---|---|---|---|----|
|          | BC4-132E   | - | - | - | + | - | - | - | - | + | - | - | + | + | - | 4  |
|          | BC4-132F   | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 0  |
|          | BC4-132G   | - | - | - | + | - | - | - | - | + | - | - | + | - | - | 3  |
|          | BC2-361A   | - | - | + | + | + | - | + | - | - | + | + | + | + | - | 8  |
| Family 4 | BC1-114A   | + | - | + | + | + | + | + | + | + | + | + | + | + | - | 12 |
|          | BC2-261A   | + | - | + | + | + | - | + | + | + | + | - | + | - | - | 9  |
|          | BC3-95A    | + | - | - | + | + | - | + | + | - | - | - | + | - | - | 6  |
|          | BC3-96A    | + | - | + | + | + | - | + | - | + | + | - | + | - | - | 8  |
|          | BC2-362A   | + |   | + | + | + | - | + | - | + | + | + | + | - | - | 9  |
|          | BC2-362B   | + | - | + | + | + | - | + | + | + | - | + | - | - | + | 9  |
|          | BC2F2-230A | - | - | - | + | - | - | + | + | + | - | + | - | - | + | 6  |
|          | BC2F2-230B | - | - | - | + | + | - | + | + | + | - | + | - | - | + | 7  |
|          | BC2F2-231A | + | - | + | + | - | - | - | - | - | - | - | - | - | - | 3  |
|          | BC2F2-231B | + | - | + | + | - | - | - | - | + | - | - | - | - | - | 4  |
|          | BC2F2-232A | - | - | + | - | - | - | - | + | + | - | + | - | - | + | 5  |
|          | BC2F2-232B | - | - | + | + | - | - | - | + | + | - | - | - | - | + | 5  |
|          | BC2F2-233A | + | - | - | + | - | - | - | + | - | - | + | - | - | + | 5  |
|          | BC2F2-233B | + | - | + | + | - | - | - | + | + | - | + | - | - | - | 6  |
| Family 5 | BC1-114B   | - | - | + | + | - | - | - | - | + | + | + | + | + | - | 7  |
|          | BC2-262A   | - | - | - | + | - | - | - | - | + | + | - | - | - | - | 3  |
|          | BC2-262B   | - | - | - | + | - | - | - | - | - | - | + | - | + | - | 3  |
|          | BC3-369C   | - | - | - | + | - | - | - | - | - | - | - | - | + | - | 2  |
|          | BC3-369D   | - | - | - | + | - | - | - | - | - | - | - | - | - | - | 1  |
|          | BC2-262C   | - | - | - | + | - | - | - | - | + | + | + | + | - | - | 5  |
|          | BC3-234A   | - | - | - | - | - | - | - | - | - | - | + | - | - | - | 1  |
|          | BC3F1-150A | - | - | - | - | - | - | - | - | - | - | + | - | - | - | 1  |
|          | BC3F1-150B | - | - | - | - | - | - | - | - | - | - | + | - | - | - | 1  |
|          | BC3F1-150C | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 0  |
|          | BC3F1-150D | - | - | - | - | - | - | - | - | - | - | + | - | - | - | 1  |

|          |            |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
|----------|------------|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
|          | BC3F1-150E | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 0 |
|          | BC3F1-150G | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 0 |
|          | BC3F1-150H | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 0 |
|          | BC3-234B   | - | - | - | + | - | - | - | - | - | + | + | + | - | - | 4 |
|          | BC3F1-151A | - | - | - | + | - | - | - | - | - | + | + | + | - | - | 4 |
|          | BC3F1-151B | - | - | - | + | - | - | - | - | - | + | + | + | - | - | 4 |
|          | BC3F1-151C | + | - | - | - | - | - | - | - | - | - | + | + | - | - | 3 |
|          | BC3F1-151E | - | - | - | + | - | - | - | - | - | + | - | + | - | - | 3 |
|          | BC3F1-151F | - | - | - | + | - | - | - | - | - | + | - | + | - | - | 3 |
|          | BC3F1-151I | - | - | - | - | - | - | - | - | - | + | + | + | - | - | 3 |
|          | BC3-97A    | - | - | - | + | - | - | - | - | + | + | - | + | - | - | 4 |
|          | BC3F1-141A | - | - | - | + | - | - | - | - | - | - | - | - | - | - | 1 |
|          | BC3F1-141B | - | - | - | + | - | - | - | - | + | + | - | + | - | - | 4 |
|          | BC3F1-141C | - | - | - | + | - | - | - | - | + | - | - | + | - | - | 3 |
|          | BC3F1-141D | - | - | - | - | - | - | - | - | - | + | - | - | - | - | 1 |
|          | BC3F1-141E | - | - | - | + | - | - | - | - | + | + | - | - | - | - | 3 |
|          | BC3F1-141F | - | - | - | - | - | - | - | - | + | + | - | + | - | - | 3 |
|          | BC3F1-141G | - | - | - | + | - | - | - | - | + | - | - | - | - | - | 2 |
|          | BC3F1-141H | - | - | - | + | - | - | - | - | - | + | - | + | - | - | 3 |
|          | BC3F1-141I | - | - | - | + | - | - | - | - | + | + | - | + | - | - | 4 |
|          | BC3F1-141J | - | - | - | + | - | - | - | - | - | - | - | + | - | - | 2 |
|          | BC3F1-141K | - | - | - | + | - | - | - | - | + | + | - | + | - | - | 4 |
|          | BC3F1-141L | - | - | - | + | - | - | - | - | - | + | - | + | - | - | 3 |
|          | BC2-363A   | - | - | + | + | - | - | - | - | - | + | + | - | + | - | 5 |
| Family 6 | BC1-115A   | + | - | + | - | - | + | + | + | + | + | - | + | + | - | 9 |
|          | BC2-263A   | + | - | + | - | - | - | - | - | - | + | - | - | - | - | 3 |
|          | BC3-271A   | - | - | + | - | - | - | - | - | - | + | - | - | - | - | 2 |
|          | BC3-271B   | + | - | + | - | - | - | - | - | - | - | - | - | - | - | 2 |
|          | BC2-263B   | + | - | + | - | - | + | - | + | - | - | - | - | - | - | 4 |

|          |            |   |   |   |   |   |   |   |   |   |   |   |   |   |   |    |
|----------|------------|---|---|---|---|---|---|---|---|---|---|---|---|---|---|----|
|          | BC2-263C   | + | - | + | - | - | + | + | - | + | + | - | + | + | - | 8  |
|          | BC3-98A    | + | - | - | + | - | - | - | - | - | + | + | - | + | - | 5  |
|          | BC3-98D    | + | - | - | + | - | - | + | - | - | - | - | + | - | - | 4  |
|          | BC3-98E    | + | - | + | + | - | + | - | - | - | + | - | + | + | - | 7  |
|          | BC3-99A    | - | - | + | - | - | + | + | - | + | - | - | - | + | - | 5  |
|          | BC2-364A   | - | + | - | + | - | - | - | - | + | + | + | - | + | - | 6  |
| Family 7 | BC1-115B   | + | - | - | + | - | + | - | + | + | + | - | - | + | + | 8  |
|          | BC2-264A   | - | - | - | + | - | + | - | + | + | + | - | - | - | - | 5  |
|          | BC2-264B   | + | - | - | + | - | + | - | - | + | - | - | - | + | - | 5  |
|          | BC2-264C   | + | - | - | + | - | - | - | - | - | + | - | - | + | - | 4  |
|          | BC3-100A   | + | - | - | + | - | - | - | - | - | + | - | - | - | - | 3  |
|          | BC3F1-143A | + | - | - | - | - | - | - | - | - | + | - | - | - | - | 2  |
|          | BC3F1-143B | + | - | - | + | - | - | - | - | - | - | - | - | - | - | 2  |
|          | BC3F1-143C | + | - | - | + | - | - | - | - | - | - | - | - | - | - | 2  |
|          | BC3F1-143D | + | - | - | + | - | - | - | - | - | - | - | - | - | - | 2  |
|          | BC3F1-143E | + | - | - | - | - | - | - | - | - | - | - | - | - | - | 1  |
|          | BC3F1-143F | - | - | - | + | - | - | - | - | - | + | - | - | - | - | 2  |
|          | BC3F1-143G | + | - | - | + | - | - | - | - | - | + | - | - | - | - | 3  |
|          | BC3F1-143H | + | - | - | + | - | - | - | - | - | + | - | - | - | - | 3  |
|          | BC3-100C   | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 0  |
|          | BC3-100D   | - | - | - | + | - | - | - | - | - | - | - | - | + | - | 2  |
|          | BC3-100E   | - | - | - | + | - | - | - | - | - | + | - | - | - | - | 2  |
|          | BC3-100F   | + | - | - | + | - | - | - | - | - | - | - | - | - | - | 2  |
| Family 8 | BC1-190    | + | + | - | + | + | - | - | + | + | + | + | + | + | + | 11 |
|          | BC2-265A   | - | + | - | + | + | - | - | - | + | + | + | - | + | - | 7  |
|          | BC3-101A   | - | - | - | - | - | - | - | - | + | + | - | - | - | - | 2  |
|          | BC3-101B   | - | + | - | - | - | - | - | - | + | - | - | - | + | - | 3  |
|          | BC3-101C   | - | - | - | - | + | - | - | - | + | - | - | - | - | - | 2  |
|          | BC2-265B   | - | - | - | + | - | - | - | - | - | + | + | + | + | + | 6  |

|  |            |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
|--|------------|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
|  | BC2-265D   | + | - | - | - | + | + | - | + | + | + | + | - | + | + | 9 |
|  | BC2-265E   | + | + | - | - | - | + | - | + | + | + | - | + | - | + | 8 |
|  | BC2F2-120A | - | + | - | - | - | - | - | + | + | + | - | + | - | - | 5 |
|  | BC2F2-120B | - | - | - | - | - | - | - | + | - | - | - | - | - | + | 2 |
|  | BC2F2-121A | - | - | - | - | - | + | - | - | - | - | - | + | - | + | 3 |
|  | BC2F2-121B | - | - | - | - | - | - | - | - | - | - | - | + | - | + | 2 |
|  | BC2F2-122A | - | - | - | - | - | - | - | + | - | - | - | + | - | - | 2 |
|  | BC2F2-122B | - | - | - | - | - | - | - | + | - | - | - | + | - | - | 2 |
|  | BC2F2-123A | + | + | - | - | - | - | - | + | - | - | - | - | - | - | 3 |
|  | BC2F2-123B | + | + | - | - | - | - | - | + | - | - | - | - | - | + | 4 |
|  | BC2F2-124A | + | - | - | - | - | - | - | + | + | + | - | + | - | + | 6 |
|  | BC2F2-124B | + | - | - | - | - | - | - | + | + | + | - | - | - | - | 4 |
|  | BC2F2-125A | - | - | - | - | - | + | - | + | - | - | - | + | - | - | 3 |
|  | BC2F2-125B | - | - | - | - | - | - | - | + | - | - | - | - | - | - | 1 |
|  | BC2F2-126A | - | - | - | - | - | - | - | + | - | - | - | + | - | - | 2 |
|  | BC2F2-126B | + | + | - | - | - | - | - | + | - | - | - | + | - | + | 5 |
|  | BC2F2-127A | - | - | - | - | - | - | - | + | - | - | - | - | - | - | 1 |
|  | BC2F2-127B | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 0 |
|  | BC2F2-128C | - | - | - | - | - | + | - | - | + | + | - | + | - | - | 4 |
|  | BC2-265F   | - | + | - | + | - | - | - | + | - | + | - | + | + | + | 7 |
|  | BC2-365A   | + | - | - | + | - | - | - | - | + | - | - | + | + | - | 5 |
|  | BC2-443A   | - | - | - | + | - | - | - | + | + | - | - | + | - | + | 5 |
|  | BC2-443B   | + | - | - | + | - | - | - | + | + | + | + | - | + | + | 8 |
|  | BC2-443C   | - | + | - | + | + | - | - | - | + | - | + | + | - | + | 7 |
|  | BC3-375A   | - | - | - | + | + | - | - | - | + | - | - | + | - | + | 5 |
|  | BC3-375B   | - | - | - | + | - | - | - | - | + | - | - | + | - | + | 4 |
|  | BC4-146A   | - | - | - | + | - | - | - | - | + | - | - | + | - | - | 3 |
|  | BC4-146B   | - | - | - | + | - | - | - | - | + | - | - | + | - | + | 4 |
|  | BC4-146C   | - | - | - | + | - | - | - | - | + | - | - | - | - | + | 3 |

|          |          |   |   |   |   |   |   |   |   |   |   |   |   |   |   |    |
|----------|----------|---|---|---|---|---|---|---|---|---|---|---|---|---|---|----|
|          | BC4-146D | - | - | - | + | - | - | - | - | + | - | - | + | - | + | 4  |
|          | BC4-146E | - | - | - | + | - | - | - | - | - | - | + | + | - | + | 4  |
|          | BC4-146F | - | - | - | + | - | - | - | - | + | - | + | + | - | + | 5  |
|          | BC2-444A | - | - | - | + | + | - | - | - | + | + | - | - | - | + | 5  |
|          | BC3-377A | - | - | - | + | - | - | - | - | + | + | - | - | - | + | 4  |
|          | BC3-377B | - | - | - | + | + | - | - | - | + | + | - | - | - | + | 5  |
|          | BC2-444B | - | - | - | + | + | - | - | + | - | - | + | + | - | + | 6  |
|          | BC3-434A | - | - | - | + | + | - | - | - | - | - | - | - | + | - | 3  |
|          | BC3-435A | - | - | - | + | + | - | - | + | - | - | + | - | + | + | 6  |
|          | BC3-435B | - | - | - | + | + | - | - | - | - | - | - | + | + | + | 5  |
|          | BC3-436A | - | - | - | + | + | - | - | - | - | - | + | - | + | - | 4  |
|          | BC2-444C | - | + | - | + | - | - | - | + | + | + | - | + | - | + | 7  |
|          | BC2-444D | + | - | - | + | + | - | - | + | + | + | + | - | + | + | 9  |
|          | BC3-376A | - | - | - | + | + | - | - | - | + | - | - | - | + | - | 4  |
|          | BC3-376B | - | - | - | - | + | - | - | + | + | - | + | - | + | + | 6  |
|          | BC2-444E | + | - | - | + | + | - | - | + | + | + | - | + | - | - | 7  |
| Family 9 | BC1-192A | + | + | + | + | + | + | - | + | + | + | + | + | + | + | 13 |
|          | BC2-266A | + | + | + | + | - | + | - | + | - | + | - | - | - | - | 7  |
|          | BC3-272A | - | + | - | + | - | + | - | - | - | + | - | - | - | - | 4  |
|          | BC3-272B | + | - | + | + | - | - | - | - | - | - | - | - | - | - | 3  |
|          | BC3-272C | + | - | + | + | - | - | - | + | - | + | - | - | - | - | 5  |
|          | BC2-266B | + | + | + | + | + | + | - | - | + | + | + | + | - | - | 10 |
|          | BC2-266C | - | - | - | + | - | - | - | + | + | - | + | + | + | - | 6  |
|          | BC3-102A | - | - | - | + | - | - | - | - | + | - | + | - | + | - | 4  |
|          | BC3-103A | - | - | - | - | - | - | - | - | + | - | - | + | - | - | 2  |
|          | BC3-103C | - | - | - | + | - | - | - | - | - | - | - | - | + | - | 2  |
|          | BC3-103D | - | - | - | + | - | - | - | - | + | - | - | + | + | - | 3  |
|          | BC2-366A | - | + | + | + | - | + | - | + | + | + | + | - | + | - | 9  |
|          | BC2-366B | + | - | + | + | - | + | - | - | + | + | + | - | + | - | 8  |



[illegible]

|           |            |   |   |   |   |   |   |   |   |   |   |   |   |   |   |    |
|-----------|------------|---|---|---|---|---|---|---|---|---|---|---|---|---|---|----|
|           | BC3-105B   | - | - | - | + | + | - | - | - | + | - | - | - | - | - | 3  |
|           | BC3-273A   | - | - | - | + | - | - | - | - | + | - | - | - | - | - | 2  |
|           | BC2-368A   | - | + | + | + | + | - | - | - | - | - | - | - | + | - | 5  |
|           | BC2F2-254A | - | + | + | + | + | - | - | - | - | - | - | - | - | - | 4  |
|           | BC2F2-254B | - | + | + | + | - | - | - | - | - | - | - | - | - | - | 3  |
|           | BC2F2-255A | - | + | - | + | - | - | - | - | - | - | - | - | - | - | 2  |
|           | BC2F2-255B | - | + | + | + | - | - | - | - | - | - | - | - | - | - | 3  |
|           | BC2F2-256A | - | - | + | + | - | - | - | - | - | - | - | - | - | - | 2  |
|           | BC2F2-256B | - | - | + | + | - | - | - | - | - | - | - | - | - | - | 2  |
|           | BC2F2-258A | - | + | - | + | - | - | - | - | - | - | - | - | + | - | 3  |
|           | BC2F2-258B | - | + | - | + | - | - | - | - | - | - | - | - | + | - | 3  |
|           | BC2F2-260A | - | + | + | + | + | - | - | - | - | - | - | - | - | - | 4  |
|           | BC2F2-260B | - | + | + | + | - | - | - | - | - | - | - | - | - | - | 3  |
|           | BC2F2-260C | - | + | + | + | + | - | - | - | - | - | - | - | + | - | 5  |
|           | BC2F2-261A | - | - | + | + | - | - | - | - | - | - | - | - | - | - | 2  |
|           | BC2F2-261B | - | - | + | + | - | - | - | - | - | - | - | - | - | - | 2  |
|           | BC2-368B   | - | - | + | + | - | - | - | - | + | - | - | - | - | - | 3  |
| Family 12 | BC1-195    | + | - | + | + | + | + | + | + | + | + | + | + | + | + | 13 |
|           | BC2-269A   | + | - | + | + | - | + | - | - | + | + | + | - | - | + | 8  |
|           | BC2-269B   | + | - | + | + | - | + | - | + | + | - | + | - | + | + | 9  |
|           | BC3-370A   | - | - | + | + | - | - | - | + | - | - | + | - | + | - | 5  |
|           | BC2-269C   | + | - | + | - | - | - | + | + | + | - | + | + | + | + | 9  |
|           | BC3-106E   | + | - | + | + | - | - | - | - | - | - | + | - | + | - | 5  |
|           | BC3-106F   | - | - | + | + | - | - | - | + | + | - | - | - | + | + | 6  |
|           | BC3-427A   | - | - | + | + | - | - | + | + | + | - | - | + | + | - | 7  |
|           | BC3-472A   | + | - | + | + | - | - | - | + | + | - | - | + | + | + | 8  |
|           | BC3-472B   | + | - | + | + | - | - | - | - | + | - | + | + | + | + | 8  |
|           | BC2F1-201A | + | - | + | + | - | - | + | + | + | - | + | + | + | + | 10 |
|           | BC3-106A   | - | - | - | + | - | - | - | + | - | - | - | + | + | - | 4  |

|           |            |   |   |   |   |   |   |   |   |   |   |   |   |   |   |    |
|-----------|------------|---|---|---|---|---|---|---|---|---|---|---|---|---|---|----|
|           | BC3-106C   | + | - | + | + | - | - | + | + | - | - | + | - | - | - | 6  |
|           | BC3F1-147A | - | - | + | - | - | - | + | + | - | - | - | - | - | - | 3  |
|           | BC3F1-147B | + | - | + | + | - | - | + | + | - | - | + | - | - | - | 6  |
|           | BC3F1-147C | - | - | + | + | - | - | + | + | - | - | + | - | - | - | 5  |
|           | BC3F1-147D | - | - | + | + | - | - | + | + | - | - | - | - | - | - | 4  |
|           | BC4-136A   | + | - | - | + | - | - | - | + | - | - | - | - | - | - | 3  |
|           | BC4-136B   | - | - | + | + | - | - | - | + | - | - | - | - | - | - | 3  |
|           | BC4-136C   | + | - | + | + | - | - | + | + | - | - | - | - | - | - | 5  |
|           | BC4-136D   | + | - | + | + | - | - | + | + | - | - | + | - | - | - | 6  |
|           | BC2-369A   | + | - | + | + | + | + | + | - | + | + | - | - | + | - | 9  |
| Family 13 | BC1-243    | + | + | + | + | + | - | + | - | + | + | + | + | - | - | 10 |
|           | BC2-270A   | - | + | + | - | + | - | + | - | - | - | + | + | - | - | 6  |
|           | BC3-274A   | - | + | - | - | - | - | - | - | - | - | + | - | - | - | 2  |
|           | BC3-235A   | - | - | - | - | + | - | + | - | - | - | - | + | - | - | 3  |
|           | BC3F1-152A | - | - | - | - | + | - | + | - | - | - | - | + | - | - | 3  |
|           | BC3F1-152B | - | - | - | - | + | - | + | - | - | - | - | + | - | - | 3  |
|           | BC3F1-152C | - | - | - | - | + | - | + | - | - | - | - | + | - | - | 3  |
|           | BC3F1-152D | - | - | - | - | + | - | + | - | - | - | - | + | - | - | 3  |
|           | BC3F1-152E | - | - | - | - | + | - | + | - | - | - | - | + | - | - | 3  |
|           | BC3F1-152F | - | - | - | - | + | - | + | - | - | - | - | + | - | - | 3  |
|           | BC3F1-152I | - | - | - | - | - | - | - | - | - | - | - | + | - | - | 1  |
|           | BC3F1-152J | - | - | - | - | + | - | + | - | - | - | - | + | - | - | 3  |
|           | BC3F1-152K | - | - | - | - | + | - | + | - | - | - | - | + | - | - | 3  |
|           | BC3-235B   | - | - | + | - | + | - | + | - | - | - | - | + | - | - | 4  |
|           | BC2-573A   | + | + | + | + | + | - | + | - | + | + | + | - | - | - | 9  |
|           | BC2-573B   | + | - | + | + | + | - | + | - | - | - | + | - | - | - | 6  |
| Family 14 | BC1-300A   | + | + | + | + | + | - | + | + | + | + | + | + | + | + | 13 |
|           | BC2-271A   | + | + | + | - | - | - | - | + | - | - | - | + | - | + | 6  |
|           | BC3-107A   | + | + | + | - | - | - | - | + | - | - | - | + | - | + | 6  |

|           |          |   |   |   |   |   |   |   |   |   |   |   |   |   |   |    |
|-----------|----------|---|---|---|---|---|---|---|---|---|---|---|---|---|---|----|
|           | BC2-271B | + | - | - | + | + | - | + | - | + | - | - | - | - | + | 6  |
|           | BC3-236B | - | - | - | - | - | - | - | - | + | - | - | - | - | + | 2  |
|           | BC2-271C | - | + | + | - | - | - | - | + | + | + | + | - | - | + | 7  |
| Family 15 | BC1-300B | - | + | - | + | + | + | + | + | + | + | + | + | - | - | 10 |
|           | BC2-272A | - | + | - | - | - | - | - | - | - | + | - | + | - | - | 3  |
|           | BC3-108A | - | + | - | - | - | - | - | - | - | - | - | + | - | - | 2  |
|           | BC3-109A | - | + | - | - | - | - | - | - | - | + | - | - | - | - | 2  |
|           | BC2-371A | - | - | - | + | - | + | - | + | - | + | - | + | - | - | 5  |
|           | BC2-371B | - | - | - | + | - | - | - | + | + | + | - | - | - | - | 4  |
|           | BC3-373A | + | - | - | + | - | - | - | + | - | + | - | - | - | - | 4  |
|           | BC3-373B | + | - | - | + | - | - | - | - | - | + | - | - | - | - | 3  |
| Family 16 | BC1-300C | - | + | + | + | + | + | + | + | + | + | + | + | + | + | 13 |
|           | BC2-372A | - | - | + | + | - | + | + | + | - | + | - | + | + | + | 9  |
|           | BC2-372B | - | - | - | - | + | - | + | + | - | - | - | - | + | - | 4  |
| Family 17 | BC1-302  | - | + | + | + | - | + | - | - | + | + | + | + | + | + | 10 |
|           | BC2-575A | - | - | - | + | - | + | - | - | + | - | + | + | - | + | 6  |
|           | BC2-575B | - | - | + | + | - | + | - | - | + | - | + | + | + | + | 8  |
|           | BC3-447A | - | - | + | + | - | + | - | - | - | - | - | - | - | + | 4  |
|           | BC3-447B | + | - | + | + | - | + | - | - | + | - | - | + | + | + | 8  |
|           | BC3-447C | - | - | + | - | - | - | - | - | + | - | + | - | + | - | 4  |
|           | BC3-447D | - | - | + | - | - | + | - | - | - | - | + | + | - | - | 4  |
|           | BC3-447E | - | - | + | - | - | + | - | - | - | - | - | + | + | + | 5  |
|           | BC3-447F | - | - | - | - | - | - | - | - | + | - | - | + | - | + | 3  |
|           | BC3-447G | - | - | + | + | - | + | - | - | - | - | + | - | - | - | 4  |
|           | BC3-447H | - | - | - | + | - | + | - | - | + | - | - | + | - | + | 5  |
|           | BC2-575D | - | + | + | + | - | - | - | - | + | + | + | - | - | - | 6  |
| Family 18 | BC1-303  | + | + | + | + | + | - | + | - | + | + | + | - | + | - | 10 |
|           | BC2-275A | - | - | - | + | + | - | + | - | + | + | - | - | - | - | 5  |
|           | BC3-110A | - | - | - | - | + | - | + | - | + | + | - | - | - | - | 4  |

|           |            |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
|-----------|------------|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
|           | BC4-116A   | - | - | - | - | - | - | - | - | - | + | - | - | - | - | 1 |
|           | BC3-111A   | - | - | - | + | + | - | + | - | + | - | - | - | - | - | 4 |
|           | BC4-115A   | - | - | - | - | + | - | + | - | - | - | - | - | - | - | 2 |
|           | BC2-275D   | + | + | - | - | + | - | + | - | + | + | + | - | + | - | 8 |
|           | BC2-275E   | - | + | - | + | + | - | + | - | + | + | + | - | + | - | 8 |
|           | BC2-374A   | + | - | + | - | + | - | + | - | + | - | + | - | + | - | 7 |
| Family 19 | BC1-304    | + | - | - | + | - | + | + | + | - | + | + | - | + | + | 9 |
|           | BC2-276A   | - | - | - | + | - | - | + | + | - | + | + | - | - | - | 5 |
|           | BC3-372A   | - | - | - | - | - | - | - | + | - | - | + | - | - | - | 2 |
|           | BC3-372B   | - | - | - | + | - | - | + | + | - | + | + | - | - | - | 5 |
|           | BC2-276B   | + | - | - | + | - | - | + | - | - | - | + | - | + | - | 5 |
|           | BC3-374A   | - | - | - | + | - | - | + | - | - | - | + | - | - | - | 3 |
|           | BC4-144A   | - | - | - | - | - | - | - | - | - | - | + | - | - | - | 1 |
|           | BC4-144B   | - | - | - | - | - | - | - | - | - | - | + | - | - | - | 1 |
|           | BC4-144C   | - | - | - | - | - | - | + | - | - | - | + | - | - | - | 2 |
|           | BC4-144D   | - | - | - | + | - | - | - | - | - | - | - | - | - | - | 1 |
|           | BC4-144E   | - | - | - | - | - | - | - | - | - | - | + | - | - | - | 1 |
|           | BC4-144F   | - | - | - | + | - | - | - | - | - | - | + | - | - | - | 2 |
|           | BC4-144G   | - | - | - | + | - | - | + | - | - | - | - | - | - | - | 2 |
|           | BC4-144H   | - | - | - | + | - | - | - | - | - | - | - | - | - | - | 1 |
|           | BC3-374B   | - | - | - | + | - | - | - | - | - | - | - | - | + | - | 2 |
|           | BC2-276C   | + | - | - | + | - | - | - | + | - | + | - | - | + | + | 6 |
|           | BC3-112A   | + | - | - | + | - | - | - | + | - | - | - | - | + | - | 4 |
|           | BC3F1-149A | + | - | - | + | - | - | - | + | - | - | - | - | + | - | 4 |
|           | BC3F1-149B | - | - | - | + | - | - | - | + | - | - | - | - | + | - | 3 |
|           | BC3F1-149C | + | - | - | + | - | - | - | + | - | - | - | - | + | - | 4 |
|           | BC3F1-149D | - | - | - | + | - | - | - | - | - | - | - | - | + | - | 2 |
|           | BC3F1-149E | + | - | - | + | - | - | - | + | - | - | - | - | - | - | 3 |
|           | BC3F1-149F | + | - | - | + | - | - | - | + | - | - | - | - | + | - | 4 |

|           |             |   |   |   |   |   |   |   |   |   |   |   |   |   |   |    |
|-----------|-------------|---|---|---|---|---|---|---|---|---|---|---|---|---|---|----|
|           | BC3F1-149G  | - | - | - | + | - | - | - | + | - | - | - | - | - | - | 2  |
|           | BC3F1-149H  | + | - | - | + | - | - | - | + | - | - | - | - | + | - | 4  |
|           | BC3-113A    | - | - | - | + | - | - | - | - | - | - | - | - | + | - | 2  |
|           | BC4-114A    | - | - | - | + | - | - | - | + | - | - | - | - | + | - | 3  |
|           | BC4-114B    | - | - | - | + | - | - | - | + | - | - | - | - | + | - | 3  |
|           | BC2-375A    | + | - | - | + | - | + | - | + | - | - | - | - | + | + | 6  |
|           | BC2F2-298A  | + | - | + | + | - | - | + | + | - | - | - | - | + | - | 6  |
|           | BC2F2-298B  | - | - | - | + | - | - | - | + | - | - | - | - | + | - | 3  |
|           | BC2F2-299A  | - | - | - | + | - | + | - | + | - | - | - | - | - | - | 3  |
|           | BC2F2-299B  | - | - | - | + | - | + | - | + | - | - | - | - | + | - | 4  |
|           | BC2F2-300A  | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 0  |
|           | BC2F2-300B  | - | - | - | + | - | - | - | - | - | - | - | - | + | - | 2  |
|           | BC2F2-301A  | + | - | - | + | - | - | - | - | - | - | - | - | + | - | 3  |
|           | BC2F2-301B  | + | - | - | + | - | + | - | - | - | - | - | - | - | + | 4  |
|           | BC2F2-302A  | - | - | - | + | - | - | - | - | - | - | - | - | - | - | 1  |
|           | BC2F2-302B  | - | - | - | + | - | - | - | - | - | - | - | - | - | - | 1  |
|           | BC2F2-303A  | - | - | - | - | - | - | - | + | - | - | - | - | + | - | 2  |
|           | BC2F2-303B  | + | - | - | - | - | + | - | - | - | - | - | - | - | - | 2  |
|           | BC2F2-304A  | - | - | - | + | - | + | - | + | - | - | - | - | + | + | 5  |
|           | BC2F2-304B  | - | - | - | + | - | + | - | + | - | - | - | - | + | - | 4  |
|           | BC2F2-305A  | - | - | - | + | - | + | - | - | - | - | - | - | - | - | 2  |
|           | BC2F2-306A  | - | - | - | + | - | + | - | + | - | - | - | - | + | - | 4  |
|           | BC2F2-306B  | - | - | - | + | - | + | - | - | - | - | - | - | + | + | 4  |
|           | BC2F2-307A  | - | - | - | + | - | + | - | - | - | - | - | - | + | + | 4  |
|           | BC2F2-307B  | - | - | - | + | - | - | - | + | - | - | - | - | + | - | 3  |
|           | BC2-375B    | + | - | - | + | - | - | + | + | - | + | - | - | + | - | 6  |
| Family 20 | BC1-384A    | - | - | + | + | + | - | + | + | + | + | + | + | + | + | 11 |
|           | BC2-537A    | - | - | + | + | - | - | - | - | + | - | - | - | + | - | 4  |
|           | BC2F1-249-1 | - | - | + | + | - | - | - | - | + | - | - | - | - | - | 3  |

|           |              |   |   |   |   |   |   |   |   |   |   |   |   |   |   |    |
|-----------|--------------|---|---|---|---|---|---|---|---|---|---|---|---|---|---|----|
|           | BC2F1-249-2  | - | - | + | + | - | - | - | - | - | - | - | - | + | - | 3  |
|           | BC2F1-249-3  | - | - | - | - | - | - | - | - | + | - | - | - | - | - | 1  |
|           | BC2F1-249-4  | - | - | - | + | - | - | - | - | + | - | - | - | + | - | 3  |
|           | BC2F1-249-5  | - | - | + | + | - | - | - | - | - | - | - | - | + | - | 3  |
|           | BC2F1-249-6  | - | - | - | - | - | - | - | - | + | - | - | - | + | - | 2  |
|           | BC2F1-249-7  | - | - | - | + | - | - | - | - | - | - | - | - | + | - | 2  |
|           | BC2F1-249-8  | - | - | + | + | - | - | - | - | + | - | - | - | + | - | 4  |
|           | BC2F1-249-9  | - | - | - | + | - | - | - | - | + | - | - | - | + | - | 3  |
|           | BC2F1-249-10 | - | - | - | + | - | - | - | - | + | - | - | - | + | - | 3  |
|           | BC2F1-249-11 | - | - | - | - | - | - | - | - | + | + | - | - | - | - | 2  |
|           | BC2-538A     | - | - | - | + | - | - | - | - | + | + | - | + | - | - | 4  |
|           | BC2-538B     | - | - | + | + | + | - | + | + | - | + | - | + | + | + | 9  |
|           | BC2-538C     | - | - | + | + | + | - | - | + | + | + | - | - | + | - | 7  |
| Family 21 | BC1-384B     | + | - | + | + | + | - | + | - | + | + | - | + | - | - | 8  |
|           | BC2-576A     | - | - | + | + | - | - | - | - | - | + | - | + | - | - | 4  |
|           | BC2-577B     | - | - | + | + | - | - | - | - | + | + | - | - | - | - | 4  |
| Family 22 | BC1-384C     | - | - | + | + | - | + | - | + | + | - | + | + | + | + | 9  |
|           | BC2-578B     | - | - | - | + | - | - | - | - | - | - | + | + | - | + | 4  |
|           | BC2-578C     | - | - | + | + | - | + | - | + | + | - | + | - | - | - | 6  |
|           | BC2-578D     | - | - | - | + | - | - | - | + | + | - | + | - | - | - | 4  |
| Family 23 | BC1-386A     | + | - | + | + | + | - | + | + | + | + | - | + | + | + | 11 |
|           | BC2-539A     | + | - | - | + | + | - | + | - | - | + | - | + | - | + | 7  |
|           | BC2-539B     | + | - | + | + | + | - | + | - | + | + | - | + | - | + | 9  |
|           | BC2-539C     | - | - | - | + | + | - | - | - | + | - | - | + | - | - | 4  |
|           | BC3-437A     | - | - | - | + | - | - | - | - | + | - | - | - | - | - | 2  |
|           | BC3-437B     | - | - | - | + | - | - | - | - | + | - | - | + | - | - | 3  |
|           | BC3-437C     | - | - | - | + | - | - | - | - | + | - | - | + | - | - | 3  |
|           | BC3-437D     | - | - | - | + | - | - | - | - | + | - | - | - | - | - | 2  |
|           | BC3-437E     | - | - | - | + | - | - | - | - | + | - | - | + | - | - | 3  |

|           |            |   |   |   |   |   |   |   |   |   |   |   |   |   |   |    |
|-----------|------------|---|---|---|---|---|---|---|---|---|---|---|---|---|---|----|
|           | BC3-437F   | - | - | - | - | + | - | - | - | - | - | - | - | - | - | 1  |
|           | BC3-437G   | - | - | - | + | + | - | - | - | + | - | - | + | - | - | 4  |
|           | BC3-437H   | - | - | - | + | - | - | - | - | + | - | - | - | - | - | 2  |
|           | BC3-437I   | - | - | - | + | - | - | - | - | - | - | - | + | - | - | 2  |
|           | BC2F1-207A | - | - | - | + | + | - | + | - | + | - | - | - | + | - | 5  |
|           | BC2-539D   | - | - | + | + | - | - | - | + | - | + | - | + | + | + | 7  |
| Family 24 | BC1-387A   | + | + | + | + | + | - | + | + | + | + | + | + | + | - | 12 |
|           | BC2-540A   | + | + | - | + | - | - | + | - | + | - | + | + | - | + | 8  |
|           | BC2-579A   | + | - | + | + | + | - | + | - | - | - | - | + | - | + | 7  |
|           | BC2-579B   | + | - | - | + | + | - | + | - | - | - | - | - | - | + | 5  |
|           | BC2-579C   | + | + | - | + | - | - | + | - | + | - | - | + | - | - | 6  |
| Family 25 | BC1-387B   | + | - | + | + | - | + | + | + | + | + | + | + | + | + | 12 |
|           | BC2-580A   | + | - | + | + | - | - | - | - | - | + | + | + | + | - | 7  |
|           | BC2-580B   | + | - | - | + | - | + | - | - | + | - | + | + | + | - | 7  |
| Family 26 | BC1-388A   | + | + | + | + | - | + | - | + | + | + | + | + | + | + | 12 |
|           | BC2-541A   | + | - | + | + | - | - | - | + | - | - | + | - | - | - | 5  |
|           | BC2-542A   | - | - | - | + | - | + | - | - | + | - | + | + | + | - | 6  |
|           | BC2-542C   | + | + | + | + | - | - | - | + | - | + | + | + | + | - | 9  |
|           | BC3-439A   | - | - | - | + | + | - | - | - | - | - | - | - | + | - | 3  |
|           | BC3-439B   | + | - | - | + | - | - | - | + | - | + | - | + | + | + | 7  |
|           | BC3-439C   | + | - | - | - | - | - | - | - | - | + | + | + | - | + | 5  |
|           | BC3-439D   | + | + | + | + | - | - | - | - | - | - | + | - | - | + | 6  |
|           | BC3-439E   | + | - | + | - | - | - | - | + | - | - | + | - | - | + | 5  |
|           | BC3-439F   | + | - | - | + | - | - | - | - | - | - | + | - | - | + | 4  |
|           | BC3-439G   | + | + | - | + | - | - | - | + | - | - | + | - | - | + | 6  |
|           | BC3-439H   | - | - | - | + | - | + | - | + | - | + | - | + | - | + | 6  |
| Family 27 | BC1-389A   | + | - | - | + | - | - | + | - | - | - | + | + | - | - | 5  |
|           | BC2-543A   | - | - | - | + | - | - | - | - | - | - | - | + | - | - | 2  |
|           | BC2-543B   | + | - | - | - | - | - | + | - | - | - | - | - | - | - | 2  |



|           |            |   |   |   |   |   |   |   |   |   |   |   |   |   |   |    |
|-----------|------------|---|---|---|---|---|---|---|---|---|---|---|---|---|---|----|
|           | BC2-543C   | + | - | - | - | - | - | + | - | - | - | + | - | - | - | 3  |
| Family 28 | BC1-389B   | - | - | + | + | + | - | + | - | + | + | + | - | - | - | 7  |
|           | BC2-544A   | - | - | + | + | - | - | - | - | + | - | + | - | - | - | 4  |
|           | BC2-635A   | - | - | + | + | - | - | - | - | + | + | + | - | - | - | 5  |
|           | BC2-635B   | - | - | + | + | + | - | + | - | - | + | + | - | - | - | 6  |
|           | BC3-477A   | - | - | - | + | + | - | - | - | - | - | - | - | - | - | 2  |
|           | BC3-477C   | - | - | + | + | + | - | + | - | + | - | + | - | - | - | 6  |
|           | BC3-477E   | - | - | + | + | - | - | - | - | - | - | + | - | - | - | 3  |
|           | BC2F1-213A | - | - | - | + | + | - | + | - | - | + | + | - | - | - | 5  |
|           | BC3-451C   | - | - | + | + | - | - | - | - | - | - | - | - | - | - | 2  |
| Family 29 | BC1-390A   | - | - | + | + | + | + | + | - | + | + | - | + | + | - | 9  |
|           | BC2-545A   | - | - | + | + | + | - | - | - | + | + | - | - | + | - | 6  |
|           | BC2-546C   | - | - | + | + | + | - | + | - | + | + | - | - | - | - | 6  |
|           | BC3-474A   | - | - | + | + | + | - | - | - | + | - | - | - | - | - | 4  |
|           | BC3-474B   | - | - | + | + | - | - | + | - | + | + | - | - | - | - | 5  |
|           | BC3-440A   | - | - | + | + | + | - | - | - | + | + | - | - | - | - | 5  |
|           | BC3-440B   | - | - | + | - | - | - | + | - | + | - | - | - | - | - | 3  |
|           | BC3-440C   | - | - | - | + | + | - | + | - | + | - | - | - | - | - | 4  |
|           | BC3-441A   | - | - | - | + | + | - | + | - | + | - | - | - | - | - | 4  |
|           | BC3-441B   | - | - | + | - | + | - | + | - | - | - | - | - | - | - | 3  |
|           | BC3-441C   | - | - | + | + | + | - | + | - | + | + | - | - | - | - | 6  |
|           | BC3-441D   | - | - | + | + | - | - | - | - | - | + | - | - | - | - | 3  |
|           | BC3-441E   | - | - | + | + | + | - | - | - | + | - | - | - | - | - | 4  |
|           | BC2-702A   | - | - | - | + | + | - | - | - | - | + | - | + | + | - | 5  |
| Family 30 | BC1-392A   | + | + | + | + | + | - | + | + | + | + | + | + | + | + | 13 |
|           | BC2-547A   | - | - | - | + | + | - | + | - | + | + | + | + | - | + | 8  |
|           | BC2-636A   | + | - | + | + | - | - | - | - | + | + | - | + | + | - | 7  |
|           | BC2-636B   | - | - | + | + | - | - | + | + | + | - | - | - | + | + | 7  |
| Family 31 | BC1-393A   | + | - | + | + | - | - | + | + | + | + | + | - | + | + | 10 |

|           |             |   |   |   |   |   |   |   |   |   |   |   |   |   |   |    |
|-----------|-------------|---|---|---|---|---|---|---|---|---|---|---|---|---|---|----|
|           | BC2-548A    | + | - | - | + | - | - | + | + | + | - | - | - | + | - | 6  |
|           | BC2-548B    | - | - | - | + | - | - | - | - | - | + | - | - | + | + | 4  |
|           | BC3-442A    | - | - | - | + | - | + | - | - | - | + | - | - | + | - | 4  |
|           | BC3-442B    | - | - | - | - | - | - | - | - | - | + | - | - | + | + | 3  |
|           | BC3-442C    | - | - | - | + | - | - | - | - | - | - | - | - | + | + | 3  |
|           | BC3-442D    | - | - | - | + | - | - | - | - | - | + | - | - | - | - | 2  |
|           | BC2F1-210A  | - | - | - | + | - | - | - | - | - | + | - | - | + | + | 4  |
|           | BC2F1-210B  | - | - | - | + | - | - | - | - | - | - | - | - | + | - | 2  |
|           | BC2F1-210C  | - | - | - | - | - | - | - | - | - | + | - | - | + | + | 3  |
|           | BC2F1-210D  | - | - | - | + | - | - | - | - | - | + | - | - | + | + | 4  |
|           | BC2-548C    | + | - | + | - | - | - | + | + | + | - | - | - | - | - | 5  |
|           | BC2-548D    | + | - | - | - | - | - | - | - | + | - | + | - | - | - | 3  |
|           | BC2F1-248-1 | + | - | - | - | - | - | - | + | + | - | + | - | - | - | 4  |
|           | BC2F1-248-2 | + | - | - | - | - | - | - | - | + | - | + | - | - | - | 3  |
|           | BC2F1-248-3 | - | - | - | - | - | - | - | - | + | - | - | - | - | - | 1  |
|           | BC2F1-248-4 | - | - | - | - | - | - | - | - | + | - | - | - | - | - | 1  |
|           | BC2F1-248-5 | + | - | - | - | - | - | - | - | + | - | - | - | - | - | 2  |
|           | BC2F1-248-6 | - | - | - | - | - | - | - | - | + | - | + | - | - | - | 2  |
|           | BC2F1-248-7 | + | - | - | - | - | - | - | - | - | - | + | - | - | - | 2  |
|           | BC2F1-248-8 | + | - | - | - | - | - | - | - | + | - | + | - | - | - | 3  |
|           | BC2-703A    | + | - | - | + | - | - | - | + | - | - | - | - | + | - | 4  |
|           | BC2-703B    | + | - | + | + | - | - | + | - | + | - | - | - | - | - | 5  |
| Family 32 | BC1-395A    | - | + | - | + | + | + | + | + | + | + | + | - | + | + | 11 |
|           | BC2-637A    | - | + | - | + | + | + | + | - | - | + | + | - | + | - | 8  |
|           | BC2-637B    | + | + | - | + | + | + | + | + | - | - | + | - | - | - | 7  |
|           | BC3-452A    | - | + | - | + | - | + | - | - | - | - | + | - | - | - | 4  |
|           | BC3-452B    | + | - | - | + | - | + | - | - | - | - | - | - | - | - | 3  |
|           | BC3-452C    | + | - | - | + | - | + | + | + | - | - | + | - | - | - | 6  |
|           | BC3-452F    | - | + | - | - | + | - | + | - | - | - | + | - | - | - | 4  |

|           |            |   |   |   |   |   |   |   |   |   |   |   |   |   |   |    |
|-----------|------------|---|---|---|---|---|---|---|---|---|---|---|---|---|---|----|
|           | BC2F1-216A | - | + | - | - | + | - | + | + | - | - | + | - | - | - | 5  |
|           | BC2-637C   | - | + | - | + | + | + | + | - | - | + | + | - | - | + | 8  |
| Family 33 | BC1-395B   | - | - | + | - | + | - | + | - | + | + | + | - | + | - | 7  |
|           | BC2-549A   | - | - | + | - | + | - | - | - | + | - | - | - | + | - | 4  |
|           | BC3-378A   | - | - | + | - | + | - | - | - | - | - | - | - | - | - | 2  |
|           | BC3-378B   | - | - | - | + | - | - | - | - | - | - | - | - | - | - | 1  |
|           | BC2-549B   | - | - | + | - | + | - | + | - | + | + | + | - | + | - | 7  |
|           | BC2-549C   | + | - | + | - | + | - | + | - | + | - | - | - | + | - | 6  |
|           | BC2-549D   | - | - | + | - | - | - | + | - | + | + | + | - | - | - | 5  |
|           | BC2-550A   | - | - | - | - | + | - | + | - | + | - | - | - | + | - | 4  |
|           | BC2-550C   | - | - | + | - | - | - | - | - | + | + | - | - | + | - | 4  |
|           | BC2-550D   | - | - | + | - | + | - | - | - | - | + | - | - | + | - | 4  |
| Family 34 | BC1-396A   | + | + | + | + | - | - | - | + | + | + | + | + | + | + | 11 |
|           | BC2-551A   | + | - | + | - | - | - | - | - | + | - | + | + | + | + | 7  |
|           | BC2-551B   | + | + | - | + | - | - | - | + | - | - | - | + | + | - | 6  |
|           | BC2-551C   | + | + | - | - | - | - | - | + | - | - | + | - | - | - | 4  |
| Family 35 | BC1-397A   | + | - | + | + | + | - | + | + | + | + | - | + | + | - | 10 |
|           | BC2-552A   | + | - | + | + | - | - | + | + | + | - | - | - | + | - | 7  |