Genetic analysis of crosses between hexaploid bread (*Triticum aestivum* L.) and tetraploid durum wheat (*T. turgidum* spp *durum*)

A Thesis submitted by

Sriram Padmanaban, MSc (Agri)

For the award of

Doctor of Philosophy

2018

ABSTRACT

An ability to access and then transfer genes from bread wheat into durum wheat and vice-versa is an excellent way to improve the genetic architecture of these two closely related but a different ploidy level wheat species. Bread wheat has number of desirable characters such as partial resistance to crown rot and head blight that can complement durum wheat. Durum wheat has a number of desirable traits such as seed weight, seed colour, and nematode tolerance that can complement bread wheat. Therefore, developing hexaploid/tetraploid crosses can be one of the useful breeding techniques to addresses bread and durum wheat improvement for yield, pest and disease resistance. Before screening for any traits that have been incorporated from bread and durum wheat into hexaploid/tetraploid derived lines, it is necessary to understand how these inter-ploidy crosses are different with regards to inheritance of the nuclear and cytoplasmic genomes. Thus the present study aimed to screen nuclear and cytoplasmic genome inheritance of different hexaploid/tetraploid wheat crosses.

To determine the proportion of nuclear genome inheritance from either parent of the hexaploid/tetraploid derived wheat lines, high-density polymorphic DArT markers and cytological genomic and fluorescence *in situ* hybridisation were employed. To investigate the cytoplasmic mitochondrial inheritance, targeted cytochrome maturation genes *ccmfn*, *ccmfc* and *nad*3 genes of bread and durum wheat were sequenced using Sanger sequencing. Different hexaploid/tetraploid crosses were established following different breeding techniques, i.e., reciprocal crosses, crosses involving different hexaploid and tetraploid cultivars, crosses made at different time points; and crosses involving a bread wheat cultivar with an introgressed 2G segment. Retention of D-genome chromosomes, proportion of chromosome A and B genome alleles inherited, and how introgressed 2G segment of bread wheat cultivars inherits when combined with tetraploid durum wheat were discussed in different respective research chapters. This thesis also has an additional chapter summarising the maternal inheritance of cytoplasmic DNA in polyploidy crosses. Overall this study has illustrated how hexaploid/tetraploid wheat crosses can be used in the commercial plant breeding programs for bread and durum wheat improvement.

Certification of Thesis

This Thesis is the work of Sriram Padmanaban except where otherwise acknowledged, with the majority of the authorship of the papers presented as a Thesis by Publication undertaken by the Student. The work is original and has not previously been submitted for any other award, except where acknowledged.

Principal Supervisor: **Dr. Anke Martin**

Associate Supervisor: **Professor Mark W. Sutherland**

Associate Supervisor: **Dr. Noel L. Knight**

Student and supervisors signatures of endorsement are held at the University.

Acknowledgements

Postgraduate research degree requires a strong support, motivation and inspiration throughout the tenure of study in order to be successful. There are many people whom I owe the greatest debt of gratitude. First and foremost are my supervisors Dr Anke Martin, Prof Mark W. Sutherland and Dr Noel L. Knight. This project would not have been achieved without the guidance and expertise that Anke Martin has offered. I would like express my sincere thanks to her for giving her time to offer valued opinions, suggestions, and support throughout my PhD. I highly appreciate her efforts and patience in moulding me as researcher and guiding me through the proper route for writing this thesis. Mark's support throughout the studies and his willingness to dedicate his time by continuously providing suggestions and comments even when he was retired are highly appreciated. I would like to thank Noel for his encouragement and support for my studies.

Special thanks go to Dr Peng Zhang for her kind assistance and support to carry out part of my research work in her cytology lab, Plant Breeding Institute, University of Sydney. I would particularly like to thank her for generously giving her time and providing me with materials and access to various facilities. I would like to express my gratitude to the Action Learning Group especially to Prof John Thompson and Dr Kirsty Owen for giving me valuable comments and suggestions to improve my manuscripts, abstracts and presentations. I also like to thank Dr Barbara Harmes at Open Access College, USQ for helping me to improve my academic English language.

I wish to express my appreciation to Rachel King for her assistance as a USQ statistics consultant. Special thanks go to Saman Seneweera for all his support, encouragement, enlightenment and motivation to achieve my goal successfully. I would also like to thank Kathleen L. Farquharson (Plant editor) for her time going through the manuscript for editing and proofreading.

This PhD project was partly funded by Grain Research & Development Corporations and the study was funded by an Australian Postgraduate Award (APA). I thank the Australian Commonwealth Government for funding my tuition fees under the tuition fees offset scheme through the Research Training Scheme (RTS)/Research Training Program (RTP). I want to thank University of Southern Queensland for providing me with support, equipment, research facilities and financial assistance, which allowed me to attend the 9th

International wheat conference in Sydney and the 20th European Association for Research on Plant Breeding (EUCARPIA) General Congress in Zürich, Switzerland.

Next, on a personal note, I would like to thank my friends and family for their support motivation and encouragement throughout my studies, especially during dispiriting moments. Above all others, for many year's mother and father provided me with care, support and unconditional love, and both emotional and financial support. Without their assistance, I would have never reached to the point of postgraduate research, so I am always indebted to them. I also want to thank my friends and colleagues Barsha Poudel, Dananjali Gamage, Joseph Berry, Kiruba Shankari and Michael Thompson who came all the way throughout my studies and helped me whenever I was in need.

Finally, I wish to express my thanks to Professor Gavin Ash (Director Centre for Crop Health) for support and financial assistance from the centre. I extend my sincere thanks to Dr Dante Adorada who welcomed me to his peanut research group and employed me as a technical officer.

Statement of Authorship

Review article. Pentaploid Wheat Hybrids: Applications, Characterisation, and Challenges. **Padmanaban S**, Zhang P, Hare RA, Sutherland MW and Martin A. **Frontiers in Plant Science,** 8:358. doi: 10.3389/fpls.2017.00358

Sriram Padmanaban (SP) contributed 70% of the conception and design, literature collection, interpretation of literature, writing and drafting. Anke Martin (AM) contributed 10% to interpretation of literature, writing, drafting and final editorial input. Mark W. Sutherland (MWS) contributed 10% to interpretation of literature, writing and drafting and final important editorial input. Peng Zhang (PZ) and Ray A. Hare contributed 5% each towards final editorial input.

Chapter 2 A Cytological and molecular analysis of D-genome chromosome retention following F_2-F_6 generations of hexaploid X tetraploid wheat crosses. **Padmanaban S**, Sutherland MW, Knight NL, and Martin A. "**Crop and Pasture Science**" 69, 121-130. doi.org/10.1071/CP17240.

SP contributed 65% of the conception and design, data collection, analysis and interpretation, writing and drafting. AM contributed 15% to the conception and design, interpretation of data, writing, drafting and final important editorial input and made the initial crosses used in the study. MWS and Noel L. Knight (NK) contributed 5% each towards interpretation of literature, writing and drafting and final important editorial input. Peng Zhang (PZ) contributed 10% of experimental design, data collection, drafting and final important editorial input.

Chapter 3 Genome inheritance in populations derived from hexaploid/tetraploid and tetraploid/hexaploid wheat crosses. **Padmanaban S**, Sutherland MW, Knight NL, and Martin A. "**Molecular Breeding**" 37:48. doi: 10.1007/s11032-017-0647-3.

SP contributed 75% to the conception and design, data collection, analysis and interpretation, writing and drafting. AM was responsible for 15% of the conception and experimental design, interpretation of data, writing, drafting and final important editorial input. MWS and NK contributed 5% each towards writing and drafting and final important editorial input.

Chapter 4. Association between presence of *Triticum timopheevii* introgression and D-genome retention in hexaploid/tetraploid wheat crosses. Padmanaban S, Zhang P, Sutherland MW, and Martin A. "Molecular Breeding" 38:84. doi: 10.1007/s11032-018-0838-6

SP contributed 70% of the conception and design, data collection, analysis and interpretation, writing and drafting. AM contributed 20% towards the conception and design, interpretation of data, writing, drafting and final important editorial input. MWS contributed 5% towards writing and drafting and final important editorial input PZ contributed 5% of the experiment design and data collection.

List of publications and submitted articles

Padmanaban S, Zhang P, Hare RA, Sutherland MW and Martin A (2017) Pentaploid Wheat Hybrids: Applications, Characterisation, and Challenges. **Frontiers in Plant Science.** 8:358. doi: 10.3389/fpls.2017.00358

Padmanaban S, Sutherland MW, Knight NL, and Martin A (2017) Genome inheritance in populations derived from hexaploid/tetraploid and tetraploid/hexaploid wheat crosses. **Molecular Breeding** 37:48. doi: 10.1007/s11032-017-0647-3

Padmanaban S, Zhang P, Sutherland MW, Knight NL and Martin A (2018) A Cytological and molecular analysis of D-genome chromosome retention following F_2-F_6 generations of hexaploid X tetraploid wheat crosses. **Crop and Pasture Science**. 69, 121-130. doi.org/10.1071/CP17240.

Padmanaban S, Zhang P, Sutherland MW and Martin A (2018) Association between presence of *Triticum timopheevii* introgression and D-genome retention in hexaploid/tetraploid wheat crosses. **Padmanaban S**, Zhang P, Sutherland MW, and Martin A. "**Molecular Breeding**" 38:84. doi: 10.1007/s11032-018-0838-6

List of Conferences attended

Padmanaban S, Sutherland MW, Knight NL, and Martin A "Inheritance of A, B and D chromosomes in a F_2 cross between *Triticum aestivum* and *T. turgidum* spp. *durum* and its reciprocal. **Poster PO30** presented under Theme 7: Wheat Improvement by Biotechnology and Genomics IWC2015 9Th International Wheat Conference, Sydney, Australia.

Padmanaban S, Zhang P, Sutherland MW, knight NL, and Martin A "Cytological and molecular analysis of D-genome retention in hexaploid/tetraploid cross" **Poster flash presentation** on "Variation in the D-genome chromosomes of hexaploid/tetraploid wheat crosses analysed utilising cytological and molecular markers". 20th EUCARPIA (European Association for research on Plant Breeding) General congress, ETH Zürich, Switzerland.

Padmanaban S, Zhang P, Sutherland MW, Knight NL, and Martin A "Pentaploid wheat hybrids prospects for future wheat breeding program" Abstract and oral presentation in a National seminar on "Conventional and molecular strategies for sustainable agriculture" held in Dept. of Genetics and Plant Breeding Annamalai University, Tamilnadu India.

TABLE OF CONTENT

Chapter 1

General Introduction

The global population is estimated to reach 9.6 billion by mid-2050. With the growing population, the demand for food, especially for cereals, is also expected to grow. Key cereal crops such as maize, wheat, and rice, are vital for feeding the global population. In order to manage the global food demand, the current production of cereal crops needs to be increased by 940 million tonnes to reach 3 billion tonnes in the next 30 years (Gao et al., 2016). Among the key cereal crops, wheat is considered the second most important after rice. The success of the wheat crop partly results from its adaptability to a wider range of growing environments than many other cereal crops. Wheat also contributes essential amino acids, minerals, vitamins, beneficial phytochemicals and dietary fiber to the human diet, and these are predominantly supplemented with whole-grain products. Because wheat is a central part of the diet for many people around the world, the significance and the necessity of bread and durum wheat production and improvement are critical and must be clearly understood. The global wheat production for the year 2015/2016 was 735 million tonnes and it is estimated to reach 751 million tonnes in 2016/2017 with an increase of 16 million tonnes (2.19%) (https://www.worldwheatproduction.com). Increasing wheat production to meet the global requirement is one of the major challenges that need to be addressed to secure food for the growing population.

High yielding bread and durum wheat cultivars are released regularly by breeders, however, changes in climatic conditions such as increasing temperature, altered patterns of precipitation and an increasing level of atmospheric carbon dioxide (CO_2) can affect the wheat crop yield significantly (Sreedhar, 2016). Furthermore, combinations of low rainfall with increased temperature or prolonged soil temperature with low humidity can facilitate the appearance of new pests and diseases which can affect the performance of even high yielding and tolerant varieties. In order to feed the growing population, improving high yielding cultivars to make them suitable for current growing conditions has to be prioritised in current plant breeding programs. Developing new high yielding wheat cultivars with improved resistance and tolerance to pests and diseases is only achievable by increasing the genetic diversity of current

breeding populations. By accelerating the genetic variability that is related to economic traits while increasing genomic resources is a key to achieving this ambitious task.

Generating genetic variability

Plant breeders may follow different breeding strategies based on their objectives and the aim of the specific plant breeding programme. For generating genetic variability different methods are available such as, wide hybridisation that includes inter or intraspecific hybridisation which helps to transfer chromosomal regions or desirable traits or genes from the wild (or) relative species to the cultivated species (Friebe et al., 1996; Sears, 1956). Mutation breeding is another strategy where seeds of whole plants are exposed to mutating chemicals (Ethyl methyl sulphate, Colchicine etc.,) or radiation (X-rays, α-rays etc.,) to develop a mutant with favorable traits (van Harten, 1998). Applying biotechnology tools such as recombinant DNA or genetically modified (GM) techniques are also employed in plant breeding for crop improvement (Hsu et al., 2014). Although many hybridisation tools are available to increase the genetic variability, the goal of this thesis is to transfer genetic information from one wheat species to another through inter-ploidy hybridisation.

Meiotic cell division in interploidy wheat hybrids

Formation of haploid gametes usually occurs through (i) the process of DNA replication and (ii) two successive nuclear divisions (meiotic prophase division I and meiotic prophase division II). The first division includes the pairing of homologous chromosomes, recombination, and segregations, i.e. shuffling of the paternal and maternal genome and reducing to half the chromosome number. However, the second meiotic division is similar to mitosis, involving the separation of sister chromatids and giving rise to four haploid daughter cells. Thus as Kleckner (1996) explained, meiosis I is a reduction division and meiosis II is an equational division. Meiosis is an important event that maintains genome stability and generates genetic variability and successful inheritability. Failure of normal meiosis can lead to serious genetic consequences including sterility, aneuploidy, and polyploidy (Cai & Xu, 2007; Cai etal., 2010).

In the case of hexaploid/tetraploid crosses, the hexaploid bread wheat has three genomes, i.e. A, B and D. Each genome (A, B, D) has seven pairs of chromosomes 1A to 7A, 1B to 7B and 1D to 7D, respectively and the chromosomal composition is 2n=6X=42.

And the tetraploid durum wheat only has two genomes, i.e. A and B. Each genome (A, B) has seven pairs of chromosomes 1A to 7A and 1B to 7B, respectively and the chromosomal composition is 2n=4X=28. Crossing these two different ploidy level wheat species, i.e. hexaploid and tetraploid, leads to unique ploidy level F_1 hybrids that are pentaploid. Kihara (1924) explained that the chromosomal constitution of pentaploid wheat hybrids is 2n=5x=35 which includes 14 bivalents (A and B genome) and 7 univalent (D genome). The 14 bivalent chromosomes behave normally throughout meiosis. The univalent D-genome chromosomes divide longitudinally at meiosis division I and without any division in meiosis II, chromosome arms migrate to either pole randomly. Thus the chromosomal constitution in both egg and nucleus varies between 14 and 21 depending on the combination of univalents. For example, an egg cell might contain 14 bivalent chromosomes and one D chromosome $14_{11}+ 1_1$ (for instance 1D) when it combines with a sperm cell that contains 14 bivalent and two D chromosomes $14_{11} +2_1$ (for instance 1D and 3D). The resultant F_2 progeny would be expected to have a complete set of the A and B genome together with a pair of 1D chromosomes and a univalent 3D chromosome.

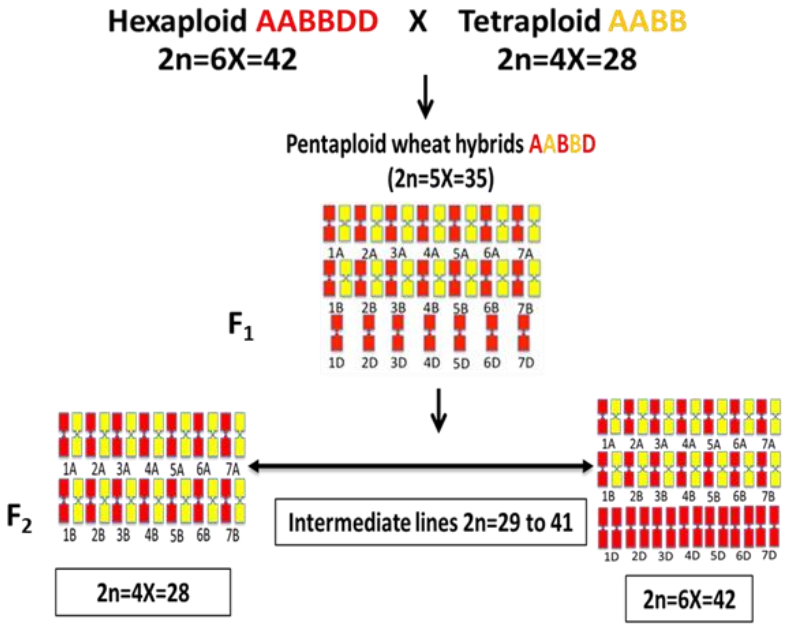


Figure 1 Schematic representation of the development of pentaploid derived wheat lines through the hexaploid and tetraploid parent.

Thus every successful F_2 seed generated from each pentaploid hybrid has various combinations of D-genome chromosomes. Some combinations can produce fertile seeds, whereas others do not survive to the next generation due to their unbalanced chromosomal nature. The current research on pentaploid wheat hybrids was summarised in our review paper (Padmanaban et al., 2017b), which also forms the part of the literature review of this thesis. This article demonstrates the advantages and disadvantages of the existing methods characterising pentaploid derived wheat lines. The potential application of pentaploid derived wheat lines in commercial plant breeding programmes and future directions for research into pentaploid wheat lines are also discussed.

"Pentaploid Wheat Hybrids: Applications, Characterisation, and Challenges". **Padmanaban S**, Zhang P, Hare RA, Sutherland MW and Martin A. **Frontiers in Plant Science,** 8:358. doi: 10.3389/fpls.2017.00358

Wheat and Introgression breeding

Wheat species with higher ploidy levels can tolerate introgressions of a whole chromosomal arm, a chromosomal segment or a gene, at a higher rate than many typical diploid species; due mainly to their buffered polyploid nature (Dubcovsky & Dvorak, 2007). This ability allows this wheat to combine with their wild or related Triticum species to increase their genetic diversity. It is desirable to generate and to maintain genetic diversity in breeding populations and released varieties in order to respond to emerging climatic and agronomic challenges or new pest or disease incursions (Dundas et al., 2007). There are several potentially useful genes that have been transferred from wild and related species and successfully deployed in commercial wheat cultivars (Friebe et al., 1996). These wheat cultivars have had or continue to have a greater impact on agriculture and food security. The most successful and well-known example is the transfer of the Rye (*Secale cereale*) chromosomal arm 1RS to wheat chromosome 1BL. This successful introgression has been extensively studied and has significantly contributed to the release of many successful wheat cultivars in the USA and Mexico. This 1R/1B translocation harbors a group of genes that confer resistance to powdery mildew (*Pm8*), leaf rust (*Lr26*), stem rust *Sr32* and yellow rust *Yr9* and also improves root and agronomic yield characters (Mago etal., 2004).

Other examples of introgression in bread wheat include *Sr*36/Pm6, from *T. timopheevii* to bread wheat chromosome 2B, *Pm13* gene from *Aegilops longissimia,* to bread wheat 3DS, *Lr28* gene from *Ae. Speltoides* to bread wheat chromosome 4AL, *Lr9* gene transferred from *Ae. umbellulata* to bread wheat chromosome 6BL and many useful genes *Sr*38/Lr37/Yr17 transferred from *Ae. ventricosa* to bread wheat chromosome 2AS (Sears 1956; Delibes et al., 1993; Friebe et al., 1996; Seah et al., 2001). These introgressions have significantly contributed to food security and are still used throughout the world.

There may be many useful resistance genes present in wild or related Triticum species, but they are often not an easy target for introgression because of the presence of fertility genes or pairing genes such as pairing homologous gene *Ph*1 or unique translocations (Jiang et al., 1993). Furthermore, there is always a trading of useful genes that are critical for expression of certain traits when the chromosomal arm or segment is translocated. Chromosomal translocations are often undetected, unless if the investigations applied proper screening techniques in studies related to inter, intra specific wheat crosses or wide hybridisation. There may be any number of useful traits introduced via alien introgression and often not characterised efficiently (Wulff & Moscou, 2014).

Furthermore, there is an incomplete understanding of the way the bread wheat cultivars with introgressed chromosomal segment transmitted to the progenies when combined with other genotypes or other species. Preferential retention of introgressed segments has been observed in past investigations (Friebe et al., 1996: Dundas et al., 2007). For example, a 2B/2G introgression that deployed gene *SrTt*3 for stem rust resistance into bread wheat from *T. timopheevi* has a strong preference to be retained as a whole translocation (Dundas et al., 2007). These investigations determined that the chromosomal segment that contains gene *SrTt*3 neither shortened nor was able to produce a recombinant through a crossover event. However, in contrast, some investigations have also proved that introgressed chromosomal segment could be shortened when crossing it with other genotype or species(Periyannan et al., 2011).

Background of the study

This Ph.D. study was part of a national research project focussing on improving crown rot resistance in existing Australian durum wheat cultivars. Australia plays a significant role in producing about 500,000 tonnes of high-quality durum wheat annually (Kneipp 2008). Currently, no durum variety has been identified that is resistant or tolerant to crown rot. However partial resistance has been reported in a number of hexaploid bread wheat lines (Martin et al., 2013). The major target of our research group is to transfer the chromosomal segments, genes that confer partial resistance to crown rot from bread wheat cultivars to the current durum wheat cultivars. To achieve this goal, it is essential to understand how hexaploid bread wheat and tetraploid durum wheat crosses inherit the genetic material in various hexaploid/tetraploid crosses.

The hexaploid/tetraploid crosses developed through this study have the potential to be employed as candidate lines for improving durum wheat for crown rot resistance. The knowledge developed through the various research experiments can serve as a guideline for plant breeders while choosing the parent material for inter-ploidy hybridisation. Furthermore, this study also addresses the importance of understanding different ploidy levels and how to combine them in the right direction for successful hybridisation. Thus this study provides additional knowledge on the comparative difference between the nuclear and cytoplasmic genome inheritance in a set of tetraploid/tetraploid and hexaploid/tetraploid wheat crosses.

Aims of the study

Despite the rich source of genetic variation that can be generated by hybridising bread and durum wheat species, this technique has not been widely used in breeding techniques, screening, and selection of lines for commercial release. The overall aim of the study is to enhance current tetraploid Australian durum lines by transferring desirable traits from Australian hexaploid bread wheat. Thus this Ph.D. dissertation focusses on understanding how the hexaploid/tetraploid crosses differ with respect to the nucleus and cytoplasmic genome inheritance from either parent by employing advanced molecular and cytological techniques. This thesis has four major research chapters; each research chapter has a different but related theme with a set of objectives explained in each chapter.

Research questions

1. At which generation do hexaploid/tetraploid derived progenies stabilise the univalent D-genome chromosomes?

The majority of previous studies have focussed on the retention of D-genome chromosomes using F_2 or F_5 progeny (Gilbert et al., 2000, Wang et al., 2005, Eberhard et al., 2010; Lanning et al., 2008) respectively. However, an observation of one generation with a limited number of lines is not sufficient to validate the fate of D-genome chromosomes in later generations. In order to understand the retention or elimination of D-genome chromosomes, it is essential to closely follow the successive generations. Following a number of generations of a particular cross by tracing the D-genome content of previous generations will also assist in determining how many generations it takes for D-genome stability.

2. Are reciprocal tetraploid/hexaploid crosses worth considering for durum wheat improvement?

Clearly there is a growing interest in developing hexaploid/tetraploid crosses for trait transfer from one species to another; however, most of the crosses were made using hexaploid as maternal parent. Only a small amount of research has been undertaken on reciprocal tetraploid/hexaploid crosses where the hexaploid is the paternal parent. This is mainly because having the higher ploidy level as a maternal parent will improve the success rate in relation to seed set and germination (Kihara 1924). For reciprocal tetraploid/hexaploid crosses, very little is known regarding the proportions of hexaploid and tetraploid derived material inherited in the A and B genomes, or the comparative retention of the D genome in the F_2 and subsequent F_3 generations.

3. Can hexaploid/tetraploid hybridisation help to transfer the introgressed 2G segment from bread wheat into durum wheat?

Many successful introgressions have been made and studied mainly on the commercial bread wheat cultivars (Jiang et al., 1993; Friebe et al., 1996) but not much consideration has been given to durum wheat. To overcome the knowledge gap, part of this Ph.D. study seeks to determine how bread wheat cultivar with introgressed 2B/2G segment inherits when combined with tetraploid durum cultivars to understand whether it is

possible to transfer the introgressed 2B/2G translocation from bread wheat to durum background via hexaploid/tetraploid crosses.

4. Maternal inheritance of cytoplasmic genome in a pair of hexaploid/tetraploid and tetraploid/hexaploid cross

Cytoplasmic organelle mitochondria inherit strictly maternally; however paternal inheritance has been witnessed in a number of inter or intraspecific hybridisations (Hattori et al., 2002; Laser et al., 1997; Nagata, 2010). Inheritance of paternal sequences and expression of novel sequences through inter or intraspecific hybridisation affects the pollen fertility in many crops including wheat. Even though cytoplasmic inheritance has been studied in a number of wide crosses in *Triticum* species, there is a knowledge gap in the literature regarding organelle inheritance in either hexaploid/tetraploid or tetraploid/hexaploid wheat crosses.

References

Cai X, Xu SS (2007) Meiosis-driven genome variation in plants. Current genomics 8 (3):151-161

Cai X, Xu SS, ZHu X (2010) Mechanism of haploidy- dependent unreductional meiotic cell division in polyploid wheat. chromosoma 119:275-285

Delibes A, Romero D, Aguaded S, Duce A, Mena M, Lopez-Braña I, Andrés M-F, Martin-Sanchez J-A, García-Olmedo F (1993) Resistance to the cereal cyst nematode (Heterodera avenae Woll.) transferred from the wild grassAegilops ventricosa to hexaploid wheat by a "stepping-stone" procedure. Theoretical and Applied Genetics 87 (3):402-408

Dubcovsky J, Dvorak J (2007) Genome plasticity a key factor in the success of polyploid wheat under domestication. Science 316 (5833):1862-1866

Dundas I, Anugrahwati D, Verlin D, Park R, Bariana H, Mago R, Islam A (2007) New sources of rust resistance from alien species: meliorating linked defects and discovery. Crop and Pasture Science 58 (6):545-549

Eberhard FS, Zang P, Lehmensiek A, Hare RA, Simpfendorfer S, Sutherland MW (2010) Chromosome Composition of an F_2 *Triticum aestivum* X *T.turgidum* spp. *durum* cross analysed by DArt markers and MCFISH. Crop and Pasture Science 61:619-624

Friebe B, Jiang J, Raupp W, McIntosh R, Gill B (1996) Characterization of wheat-alien translocations conferring resistance to diseases and pests: current status. Euphytica 91 (1):59-87

Gao L, Jia J, Kong X (2016) A SNP-based molecular barcode for characterization of common wheat. PloS one 11 (3):e0150947

Gilbert, J., Procunier, J., and Aung, T (2000). Influence of the D genome in conferring resistance to Fusarium head blight in spring wheat. Euphytica 114: 181-186.

Hattori N, Kitagawa K, Takumi S, Nakamura C (2002) Mitochondrial DNA heteroplasmy in wheat, Aegilops and their nucleus-cytoplasm hybrids. Genetics 160 (4):1619-1630

Hsu PD, Lander ES, Zhang F (2014) Development and applications of CRISPR-Cas9 for genome engineering. Cell 157 (6):1262-1278

Jiang J, Friebe B, Gill BS (1993) Recent advances in alien gene transfer in wheat. Euphytica 73 (3):199-212

Kalous J, Martin J, Sherman J, Heo H-Y, Blake N, Lanning S, Eckhoff J, Chao S, Akhunov E, Talbert L (2015) Impact of the D genome and quantitative trait loci on quantitative traits in a spring durum by spring bread wheat cross. Theoretical and Applied Genetics:1-13

Kihara H (1924) Cytologische und genetische Studien bei wichtigen Getreidearten mit besondere Rüchsicht auf das Verhalten der Chromosomen und die Sterilität in den Bastarden.Mem. Coll. Sci., Kyoto Imp. Univ. B: .1-200

Kleckner N (1996) Meiosis: how could it work? Proceedings of the National Academy of Sciences 93 (16):8167-8174

Kneipp J. (2008). Durum wheat production. State of New South Wales through NSW Department of Primary Industries.16p.

Lanning SP, Blake NK, Sherman JD, Talbert LE (2008) Variable production of tetraploid and hexaploid progeny lines from spring wheat by durum wheat crosses. Crop science 48 (1):199-202

Laser B, Mohr S, Odenbach W, Oettler G, Kück U (1997) Parental and novel copies of the mitochondrial orf25 gene in the hybrid crop-plant triticale: predominant transcriptional expression of the maternal gene copy. Current genetics 32 (5):337-347

Mago R, Spielmeyer W, Lawrence G, Ellis J, Pryor A (2004) Resistance genes for rye stem rust (*SrR*) and barley powdery mildew (*Mla*) are located in syntenic regions on short arm of chromosome. Genome 47 (1):112-121

Martin A, Simpfendorfer S, Hare R, Sutherland M (2013) Introgression of hexaploid sources of crown rot resistance into durum wheat. Euphytica 192:463-470.

Nagata N (2010) Mechanisms for independent cytoplasmic inheritance of mitochondria and plastids in angiosperms. Journal of plant research 123 (2):193-199

Padmanaban S, Zhang P, Hare RA, Sutherland MW, Martin A (2017b) Pentaploid Wheat Hybrids: Applications, Characterisation, and Challenges. Frontiers in Plant Science 8 (358). doi:10.3389/fpls.2017.00358

Periyannan SK, Bansal UK, Bariana HS, Pumphrey M, Lagudah ES (2011) A robust molecular marker for the detection of shortened introgressed segment carrying the stem rust resistance gene *Sr22* in common wheat. Theoretical and applied genetics 122 (1):1-7

Seah S, Bariana H, Jahier J, Sivasithamparam K, Lagudah ES (2001) The introgressed segment carrying rust resistance genes *Yr17*, *Lr37* and *Sr38* in wheat can be assayed by a cloned disease resistance gene-like sequence. Theoretical and Applied Genetics 102 (4):600-605. doi:10.1007/s001220051686

Sears E (1956) The transfer of leaf-rust resistance from *Aegilops umbellulata* to wheat. The transfer of leaf-rust resistance from Aegilops umbellulata to wheat

Sreedhar U (2016) Climate Change and Dynamics of Insect Pests. Dynamics of Crop Protection and Climate Change, C. Chattopadhyay and D. Prasad (eds), Studera Press.

van Harten AM (1998) Mutation breeding: theory and practical applications. Cambridge University Press,

Wulff BB, Moscou MJ (2014) Strategies for transferring resistance into wheat: from wide crosses to GM cassettes. Frontiers in plant science 5:692

Wang H, Liu D, Yan Z, Wei Y, Zheng Y (2005) Cytological characteristics of F*2* hybrids between *Triticum aestivum* L. and *T*. *durum Desf* with reference to wheat breeding. Journal of Applied Genetics 46: 365-369

Chapter2

Literature review

This thesis has a published review article entitled “Pentaploid Wheat Hybrids: Applications, Characterisation, and Challenges”. This review first focussed on recent research into the production of hexaploid/tetraploid- and tetraploid/hexaploid-derived pentaploid hybrids, mainly between hexaploid *T. aestivum* and tetraploids *T. durum*, *T. timopheevii,* and *T. dicoccoides*, and then discusses current techniques for characterising the chromosome composition of lines derived from them. This review also demonstrates the potential application of pentaploid derived wheat lines in commercial plant breeding programmes and future directions for research into pentaploid wheat lines are also discussed.

Padmanaban S, Zhang P, Hare RA, Sutherland MW and Martin A (2017) “Pentaploid Wheat Hybrids: Applications, Characterisation, and Challenges”. **Frontiers in Plant Science,** 8:358. doi: 10.3389/fpls.2017.00358.

Note: Supplementary data associated with this chapter are attached along with the article.

frontiers in Plant Science

REVIEW
published: 17 March 2017
doi: 10.3389/fpls.2017.00358

Pentaploid Wheat Hybrids: Applications, Characterisation, and Challenges

*Sriram Padmanaban[1], Peng Zhang[2], Ray A. Hare[1], Mark W. Sutherland[1] and Anke Martin[1]**

[1] Centre for Crop Health, University of Southern Queensland, Toowoomba, QLD, Australia, [2] Plant Breeding Institute, The University of Sydney, Sydney, NSW, Australia

Interspecific hybridisation between hexaploid and tetraploid wheat species leads to the development of F_1 pentaploid hybrids with unique chromosomal constitutions. Pentaploid hybrids derived from bread wheat (*Triticum aestivum* L.) and durum wheat (*Triticum turgidum* spp. *durum* Desf.) crosses can improve the genetic background of either parent by transferring traits of interest. The genetic variability derived from bread and durum wheat and transferred into pentaploid hybrids has the potential to improve disease resistance, abiotic tolerance, and grain quality, and to enhance agronomic characters. Nonetheless, pentaploid wheat hybrids have not been fully exploited in breeding programs aimed at improving crops. There are several potential barriers for efficient pentaploid wheat production, such as low pollen compatibility, poor seed set, failed seedling establishment, and frequent sterility in F_1 hybrids. However, most of the barriers can be overcome by careful selection of the parental genotypes and by employing the higher ploidy level genotype as the maternal parent. In this review, we summarize the current research on pentaploid wheat hybrids and analyze the advantages and pitfalls of current methods used to assess pentaploid-derived lines. Furthermore, we discuss current and potential applications in commercial breeding programs and future directions for research into pentaploid wheat.

Keywords: *in situ* hybridisation, interploidy crosses, pentaploid hybrids, *Triticum aestivum*, *Triticum turgidium* spp. *durum*

OPEN ACCESS

Edited by:
Ingo Schubert, Institute of Plant Genetics and Crop Plant Research, Germany

Reviewed by:
Robert Hasterok, University of Silesia in Katowice, Poland
Gabriella Linc, Centre for Agricultural Research (MTA), Hungary

****Correspondence:***
Anke Martin
anke.martin@usq.edu.au

Specialty section:
This article was submitted to Plant Genetics and Genomics, a section of the journal Frontiers in Plant Science

Received: *09 December 2016*
Accepted: *01 March 2017*
Published: *17 March 2017*

Citation:
Padmanaban S, Zhang P, Hare RA, Sutherland MW and Martin A (2017) Pentaploid Wheat Hybrids: Applications, Characterisation, and Challenges. Front. Plant Sci. 8:358. doi: 10.3389/fpls.2017.00358

INTRODUCTION

The two major wheat species, hexaploid bread wheat *Triticum aestivum* L. (2n = 6x = 42) and tetraploid durum wheat *T. turgidium* spp. *durum* (2n = 4x = 28), are commercially important wheat species globally. Hexaploid wheat has three diploid sets of seven chromosomes belonging to the A-, B-, and D-genomes (AABBDD), whereas tetraploid wheat only has two diploid sets of seven chromosomes belonging to the A- and B-genomes (AABB). Hybridisation between these two species with different ploidy levels leads to a pentaploid hybrid (AABBD) that has the chromosomal constitution of 2n = 5x = 35 (Kihara, 1924). The genetic variability that is combined from hexaploid and tetraploid wheat into a pentaploid hybrid has great potential in crop improvement (Eberhard et al., 2010; Martin et al., 2013; Kalous et al., 2015). However, while several reviews have focussed on the successful establishment of interspecific wheat hybrids (Sharma and Gill, 1983; Jiang et al., 1993; Friebe et al., 1996), little emphasis has been placed on developing efficient methods to incorporate these pentaploid hybrids into commercial breeding practices.

Although pentaploid wheat hybrids can be efficiently used in crop improvement programs, pest and disease resistance have principally been transferred into hexaploid or tetraploid wheats through alien introgression. Sharma and Gill (1983) reviewed the status of wide hybridisation and listed successful crosses that had been established between wheat and its related genera. They also focussed on the genes that had been successfully transferred from related wild species into commercial wheat cultivars up to that time. Jiang et al. (1993) discussed further advances in successful alien gene transfer from related species into cultivated bread and durum wheat. Friebe et al. (1996) comprehensively reviewed a number of important wheat-alien translocations and their potential in plant breeding for developing pest and disease resistance. Complications occur when trying to introgress traits across different wheat species. These include incompatibility between different *Triticum* species and sterility of the F_1 hybrids. Developing wheat hybrids through alien introgression is highly challenging when compared to hybridisation between domesticated inter-ploidy species such as bread and durum wheat. Recently, there has been a renewed interest in developing hexaploid/tetraploid wheat crosses to improve elite bread and durum wheat lines for a number of economically desirable characters (Martin et al., 2011, 2013; Han et al., 2014, 2016; Kalous et al., 2015).

This review first focusses on recent research into the production of hexaploid/tetraploid- and tetraploid/hexaploid-derived pentaploid hybrids, mainly between hexaploid *T. aestivum* and tetraploids *T. durum, T. timopheevii,* and *T. dicoccoides*, and then discusses current techniques for characterizing the chromosome composition of lines derived from them. In pentaploid wheat hybrids, the predominance of heterozygous loci present in their A and B genome, together with the retention of a haploid D genome, results in breeding material that has captured a high degree of genetic variation. Despite this rich source of genetic variation, there is still much to learn regarding the efficient screening, selection, and application of populations derived from pentaploid wheat hybrids in commercial breeding programs. This review will examine these challenges and consider the future potential of pentaploid wheat hybrids in crop improvement.

GENETIC VARIABILITY IN PENTAPLOID WHEAT HYBRIDS

Chromosome Morphology

Combining two or more different genomes into one cell may cause changes in chromosome morphology, including differences in the size, thickening, or lengthening of chromosomes, a phenomenon referred to as genome shock (Navashin, 1934). Genome shock has been well-documented (Matsuoka, 2011) and, in addition to changes in morphology, includes chromosomal rearrangements, gain or loss of chromosomal segments, gene activation and suppression, variations in the epigenome especially with respect to the pattern of cytosine methylation, and activation of transposons (Matsuoka, 2011). Increases in genome dosage and genes in alloploid wheat lines causes chromosomal imbalance, genome instability, and incompatibility. For example, morphological changes were observed in the satellite D chromosome in an interploidy cross in *Crepis* species. These observations indicated that there were chromosomes that lacked satellites in the F_1 progeny (Pikaard, 1999).

Chromosomal Constitution

Chromosome elimination is an essential process that takes place in subsequent generations derived from F_1 pentaploids, It may take a few generations to resolve the complex process of chromosome pairing and to give rise to a stable durum or bread wheat line. Subsequent generations derived from F_1 pentaploid wheat hybrids can be broadly classified into three groups, based on the presence or absence of D-genome chromosomes (**Figure 1**). The progeny belonging to the first group have lost all seven D-genome chromosomes ($2n = 4x = 28$); the second group consists of progeny that have intermediate numbers of D-genome chromosomes (total chromosome numbers ranging from $2n = 29$ to 41); while the third group have retained two copies of all seven D-genome chromosomes ($2n = 6x = 42$). Based on the objective of the breeding program that aims to develop bread or durum wheat lines, these three groups of pentaploid-derived wheat hybrids can by selfed or backcrossed with either parent. For example, lines belonging to the first group of hybrids can be selfed or backcrossed to a durum parent to develop elite durum lines (**Figure 1**).

To determine the general fate of D-genome chromosomes in the lines derived from F_1 pentaploids, we analyzed previous studies of pentaploid-derived populations from different generations of hexaploid/tetraploid and tetraploid/hexaploid crosses. The number of D-genome chromosomes retained was determined using cytological characterisation (Kihara, 1982; Wang et al., 2005; Eberhard et al., 2010), or molecular markers (Lanning et al., 2008; Eberhard et al., 2010; Martin et al., 2011; Kalous et al., 2015). Molecular marker data indicates the presence or absence of unique chromosomes, but fails to determine the number of copies of unique chromosomes. The cytological studies used in the analysis identified the number of copies of unique chromosomes present, but, did not distinguish individual chromosomes. For this analysis the cytological and molecular data that represent D-genome retention were collected and divided into two main groups. Group-1 includes (i) lines without any D-genome chromosomes and (ii) lines with an intermediate number of D-chromosomes, including lines which had lost both copies of at least one unique D-genome chromosome. With the latter the assumption was made that these lines would loose their D chromosomes in later generations and revert back to a tetraploid constitution. Group-2 includes lines which retained at least one copy of all seven D-genome chromosomes.

The proportion of D-genome chromosomes retained within each group was calculated for each cross (Supplementary Table 1). The main finding from the D genome retention analysis is that there is a high probability that viable progeny derived from pentaploid wheat hybrids will lose their D-genome chromosomes in subsequent generations ($P < 0.001$), resulting in stable tetraploid lines (Supplementary Table 1). Differences were

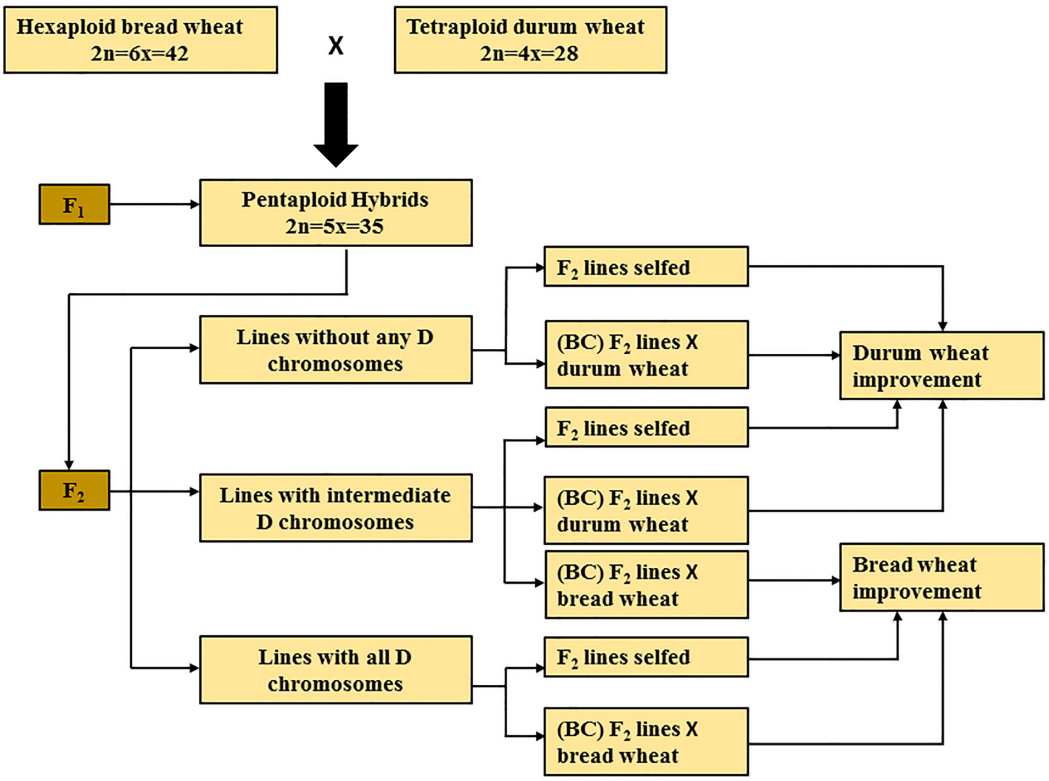


FIGURE 1 | Summary of pentaploid wheat production for bread and durum wheat improvement. BC, back crossing.

observed in the retention of D-genome chromosomes depending on the parents used in the cross. For example the hexaploid parent Choteau crossed with tetraploid parents Mountrail and Monroe, resulted in a larger number of D chromosomes being eliminated compared to the F8_Choteau/Avonlea cross (**Figure 2**). In the tetraploid/hexaploid cross involving tetraploid parent AS295 as female parent with hexaploid parents CN16 as male parent, a large proportion of D-genome chromosomes was eliminated (**Figure 2**).

Crosses involving Chinese Spring as hexaploid maternal parent and different tetraploid paternal parents retained high proportions of D-genome chromosomes. These lines are potentially useful for developing bread wheats. Conversely, Martin et al. (2011) observed that crosses involving the hexaploid variety Sunco and tetraploid breeder's line 230604 and those involving the synthetic hexaploid CPI133814 lost their D-genome chromosomes in early generations. Thus, the parental combinations used in pentaploid crosses greatly influence the degeree to which D-genome chromosomes are retained.

Relationship between Retention of Tetraploid-Derived A and B Genome and Hexaploid-Derived D Genome

Genomic analysis of generations derived from pentaploid F_1 lines indicated a significant relationship between the proportion of tetraploid-derived A and B genome retained and the retention of the D genome (Martin et al., 2011). These authors showed that the relative inheritance of chromosomes A and B alleles from bread and durum wheat differed among the hexaploid/tetraploid crosses. Lines with higher levels of durum-derived A and B chromosome segments tended to retain fewer D genome chromosomes. This implies that for characters inherited from the A and/or B genome of the hexaploid parent, pentaploid-derived lines with both low numbers of D chromosomes and the characters in question should be selected. Subsequent backcrossing will more rapidly yield elite tetraploid lines that have both lost all D genome material and inherited the targeted traits of the original hexaploid parent.

APPLICATIONS OF PENTAPLOID WHEAT HYBRIDS

Pentaploid wheat hybrids are a potential source for developing resistance to pests and diseases and for improving the level of tolerance to abiotic stresses such as salinity and metal toxicity (Munns et al., 1999; Lopes et al., 2010; Han et al., 2016). Improving quality characteristics is one of the primary concerns when developing commercial varieties suitable for human and animal consumption. For example, grain protein content, protein quality, flour color, and grain texture are key quality criteria

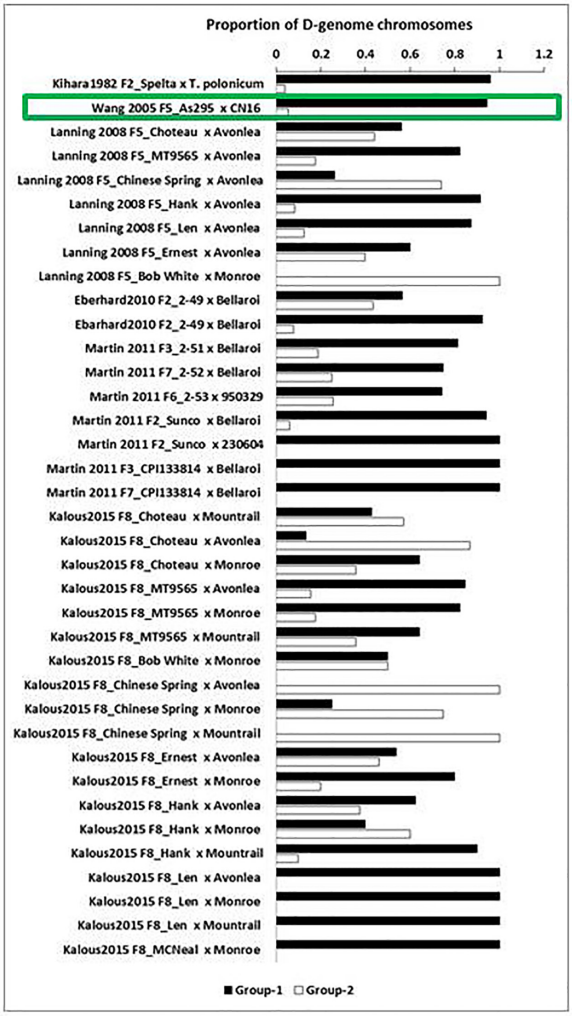


FIGURE 2 | Summary of D-genome chromosome retention in hexaploid/tetraploid and tetraploid/hexaploid crosses. Durum wheat Group-1 (lines with no or intermediate number of D chromosomes) is indicated in black and bread wheat Group-2 (lines with at least one copy of all seven D chromosomes) in white. The cross which has a tetraploid maternal parent is indicated with a green box. The proportion of D-genome chromosomes retained in each cross is indicated on the X-axis.

when developing wheat varieties for bread and pasta production (Sissons, 2008).

Disease Resistance

Crown rot, Fusarium head blight (FHB), leaf rust, stripe rust, stem rust, and powdery mildew are among the most devastating diseases of wheat that account for significant yield losses in terms of both quality and quantity (Rong et al., 2000; Bai and Shaner, 2004; Xu et al., 2013; Liu and Ogbonnaya, 2015). Crown rot disease caused by the fungus *Fusarium pseudograminearum* is considered to be one of the major constraints in durum production worldwide (Liu and Ogbonnaya, 2015). This is a chronic and severe disease in many semi-arid regions globally. In the Pacific Northwest of the USA, winter wheat crop yield reduction due to crown rot has been estimated at 35% (Smiley et al., 2005). To date, resistance to crown rot disease in durum and related tetraploid wheat species has not been identified. Partial resistance to crown rot has been identified in some bread wheat lines, such as Sunco, 2-49, and CPI133814 (Wildermuth et al., 2001; Bovill et al., 2006, 2010; Martin et al., 2015). This partial resistance in the hexaploid source is associated with multiple chromosomal regions, including 1A, 1B, 1D, 3B, and 4B in 2-49, and 2B in Sunco (Bovill et al., 2010; Martin et al., 2015). Subsequent hexaploid/tetraploid crosses were made using these partially resistant sources (2-49 and Sunco) with durum breeder's line 950329. F_6 lines were selected, based on the complete absence of D-genome chromosomes and crown rot score, and backcrossed with durum parent 950329. The BC_2F_2 progeny were assessed for crown rot severity and lines were identified with field-based resistance better than that of 2-49. The results of this study indicate that the hexaploid source of crown rot resistance was successfully introgressed into durum wheat (Martin et al., 2013).

Fusarium head blight is another important wheat disease caused by the fungus *Fusarium graminearum*, which results in a loss of yield and grain quality (Clear and Patrick, 1990). Losses due to reduced yield by FHB have been estimated to be US $2.7 billion (Matny, 2015). The production of mycotoxins by *F. graminearum* in cereal grain, particularly in wheat, is of great concern, rendering the grain unsuitable for human and animal consumption (Bottalico and Perrone, 2002). Chinese bread wheat variety Sumai3 and the two cultivars Ning8331 and 93FHB21 have been identified as resistant sources for FHB. They were crossed with the susceptible durum cultivars Stewart 63 and DT486, and the resulting F_1 pentaploid hybrids showed improved resistance to FHB compared to the durum parent (Gilbert et al., 2000).

Stripe rust caused by the fungus *Puccinia striiformis* f. sp. *tritici* is an important disease that causes major damage in both durum and bread wheat and can cause severe yield losses of more than 90% in susceptible cultivars when the weather is favorable to disease development (Chen, 2013). The Ethiopian durum wheat line accession PI 480148 has a single dominant gene *Yr53* that confers stripe rust resistance. *Yr53* was transferred into the susceptible bread wheat genotype Avocet S through pentaploid crossing (Xu et al., 2013). The progeny derived from the crosses were cytologically selected based on the presence of all seven pairs of D chromosomes ($2n = 6x = 42$) and tested with stripe rust race PST100. The progeny of the F_3 generation segregated in a 3:1 resistant: susceptible ratio, suggesting that a single dominant gene was responsible for the resistance.

Powdery mildew caused by the fungus *Blumeria graminis* f. sp. *tritici* is one of the major diseases that cause significant yield loss in wheat. Pentaploid hybrids derived from crossing susceptible hexaploid wheat cultivars Maris Nimrod and Norman with resistant tetraploid *T. dicoccoides* accession CLI060025 showed improved resistance to powdery mildew when the stable F_3 progeny were back- and top-crossed to a second hexaploid wheat (Reader and Miller, 1991). In another study, Rong et al. (2000) transferred the powdery mildew resistance gene *Pm26*, present

on chromosome 2B from an Israeli *T. dicoccoides* accession (TTD140), into the hexaploid cultivar Bethlehem.

Abiotic Stress Tolerance

Salt intolerance is a constraint that limits durum wheat production in Australia. Significant variability in saline tolerance exists in the tribe Triticeae, providing a considerable potential for transferring salt-tolerant traits into cultivated bread and durum wheat through pentaploid production (Colmer et al., 2006). Bread wheat is generally more tolerant to salinity than is durum wheat, due to the presence of salt-tolerant genes on the D-genome chromosomes (Munns et al., 1999). The major locus responsible for tolerance to salinity, *Kna1*, has been mapped to the distal end of 4DL (Lindsay et al., 2004). It has been shown that it is possible to improve the saline tolerance in durum wheat by introducing D chromosomes through wheat substitution lines. Langdon durum substitution lines, developed by Joppa and Williams (1988), have a pair of homoeologous chromosomes replaced by a pair of D chromosomes derived from the hexaploid wheat landrace Chinese Spring. These lines have opened up new avenues for developing durum wheat with improved tolerance to various abiotic stresses, including saline tolerance. A Langdon durum 4D (4B) substitution line was crossed with an Australian semi-dwarf durum wheat variety, Jandaroi, to incorporate aluminum tolerance. The chromosomal fragment of 4D was successfully introduced into the Jandaroi durum wheat, which substantially enhanced aluminum tolerance in the sister lines derived from three generations of backcrossing (Han et al., 2014). Two genes, *TaALMT1* and *TaMATE1B*, were transferred using a *Ph1* (pairing homoeologous) mutant of durum wheat through conventional breeding. The size of the 4D chromosomes introgressed from the bread wheat into durum wheat was estimated by markers, fluorescence *in situ* hybridisation (FISH), and real time quantitative PCR. The *TaALMT1* and *TaMATE1B* genes increased Al^{3+} tolerance in durum wheat and, in contrast to bread wheat, the *TaMATE1B* gene was found to be more effective in increasing Al^{3+} tolerance in durum wheat grown on acid soil (Han et al., 2016).

Quality Improvement

Understanding the molecular, chemical, and functional aspects of the quality of bread and durum wheat has significantly improved in recent decades (Shepherd, 1988; Shewry et al., 1992; Liu et al., 1996; Troccoli et al., 2000; Zhang et al., 2008). Durum wheat is mainly used for the production of pasta, semolina, couscous, and some bread products (Palumbo et al., 2000; Sissons, 2012). Durum has the hardest texture of rich yellow starchy endosperm among all of the wheat species, which makes it the most suitable for pasta production. Protein content and gluten quality are important traits in defining pasta-cooking quality; thus, the quality of the durum grain is directly associated with pasta quality (Novaro et al., 1993).

Flatbread made of durum flour is popular in the Mediterranean region and has also become popular in other countries. The inextensible dough character of durum wheat flour results in a lower loaf volume than bread wheat flour. Introgression of certain traits associated with the dough quality from bread wheat into durum wheat might improve the loaf quality of durum wheat. Palumbo et al. (2000) transferred a high molecular weight gluten portion present on chromosome 1A (*Glu-A1*) that is absent in most of the durum wheat cultivars, through interspecific hybridisation with a bread wheat variety. The resultant interspecific lines showed improved bread loaf volume (cm^3), ranging from 295 to 442.5, when compared to the tester bread wheat lines, which ranged from 390.0 to 437.5. Interspecific hybridisation involving hexaploid/tetraploid crosses has demonstrated that the resultant progeny have significantly improved grain weight, grain diameter, and grain yield (Kalous et al., 2015). Furthermore, it was suggested that introducing 1D chromosome segments and their associated protein products would improve the bread making quality of durum wheat (Sissons, 2008). Wang et al. (2005) indicated that recombinant allohexaploid lines, which had retained all seven copies of the D genome chromosomes, had enhanced protein content compared to their hexaploid parental lines. However, this study did not indicate which D genome regions are responsible for the improved bread making quality.

Allotetraploid lines derived from crossing of hexaploids/tetraploids that lack D-genome chromosomes showed improvement in storage protein content, indicating that several endosperm-protein genes on chromosomes A and B were activated in the absence of D genome chromosomes (Galili and Feldman, 1984). Furthermore they explained that the suppression of endosperm-protein genes might have occurred soon after the emergence of allohexaploid wheat, around 10,000 years ago, when the D-genome chromosomes were introduced into the tetraploid background. Potentially, these tetraploid specific endosperm-protein genes on chromosomes A and B can be re-activated in hexaploid lines that lose D chromosomes, while combining hexaploid/tetraploid and tetraploid/hexaploid crosses in pentaploid wheat production.

TECHNIQUES FOR STUDYING GENOME CONSTITUTIONS OF PENTAPLOID WHEAT HYBRIDS

Characterisation of pentaploid wheat hybrids requires techniques to identify chromosome number (e.g., of univalent D chromosomes), chromosome identity, and changes in chromosomal morphology (deletions or translocations). The following section will discuss the advantages and limitations of techniques currently available for characterizing pentaploid-derived wheat hybrids.

Cytological Characterisation of Pentaploid Wheat Hybrids

Fluorescence *in situ* hybridisation is a technique that can be used to identify chromosomes, and helps distinguish the 21 pairs of A-, B-, and D-genome chromosomes. The orginal technique involved hybridisation of radioactive-labeled DNA or RNA probes but this was subsequently replaced by fluroscence labeling of the probes (Gall and Pardue, 1969). *In situ* hybridisation enables the

identification of deletions, translocations, introgressed chromatin fragments, and translocation breakpoints (Le et al., 1989; Schwarzacher et al., 1989; Friebe et al., 1993). The physical position of known DNA sequences on the chromosomes can be visualized with the help of labeled complementary DNA strands, i.e., probes (Rayburn and Gill, 1986; Zhang et al., 2007).

Multicolor fluorescence *in situ* hybridisation (MCFISH) has been widely used for simultaneous discrimination of different genomes in polyploids, incuding cereals. This technique uses probes with dispersed repetitive DNA sequences that preferentially hybridize to the A- (BAC676D4) and D- (BAC9M13) genome chromosomes, respectively. These FISH probes are labeled with fluorescent tags of different colors and together with counterstaining of the unlabeled B-genome will distinguish the three genomes under a fluorescent microscope (Zhang et al., 2004; Komuro et al., 2013). This method has been used in pentaploid wheat hybrid studies to distinguish the A, B, and D genome chromosomes and to determine the copy number of D-genome chromosomes present in F_2 plants of hexaploid/tetraploid wheat hybrids (Eberhard et al., 2010). It is also possible to identify the individual chromosomes by using chromosome-specific repetitive probes. For instance, plasmid clone pAs1 can be used to discriminate between the individual D-genome chromosomes based on the signal patterns (Mukai et al., 1993; Koo et al., 2015). To differentiate the chromosomes of different genomes in polyploid individuals, the total genomic DNA of one parent is labeled and used as a probe, while a higher amount of unlabeled genomic DNA of the other parent is used to block the common repetitive sequences between the parents and to increase the specificity of DNA hybridisation. This technique is known as genomic *in situ* hybridisation (GISH) and can be used for studying intergenomic translocations and alien introgressions, and for discriminating genomes in polyploid cereals (Schwarzacher et al., 1989, 1992; Schubert et al., 2001; Silva and Souza, 2013).

Even though cytological techniques have improved over the past decades, there are still some disadvantages in utilizing these approaches for screening a large amount of samples. The genome size, homologous nature of diploid donor species and presence of large numbers of repetitive sequence is still challenging for wheat cytogenetics. Furthermore, cytological approaches are labor intensive, demand a high level of technical skill and require extended periods of time for assessment of multiple progeny. The time and skills required render these approaches unsuitable for use as a high-throughput screening method, as would be required for commercial breeding (Eberhard et al., 2010). However, these methods have an important role in fundamental research and in characterizing the chromosome constitution of elite pentaploid-derived lines.

Molecular Marker Technology

Unlike cytological techniques, molecular markers are not influenced by the environment or plant growth stages. Different types of molecular markers are available depending on their applications, such as hybridisation-based DNA markers, PCR-based markers, DNA chip and sequence-based markers. Applications of these markers in modern plant breeding efforts have been comprehensively discussed elsewhere (Gupta et al., 2008; Wang S. et al., 2014).

Simple sequence repeat (SSR) or microsatellite markers have been widely used and were considered as the marker of choice for many years by plant breeders analyzing interspecific hexaploid/tetraploid crosses (Lanning et al., 2008). SSR markers are readily reproducible and can be used to distinguish the presence or absence of unique chromosomes. Most SSRs are co-dominant markers and can be used to distinguish genotypes based on the size of alleles and are thus also useful for validating F_1 hybrids. When co-dominant at a particular locus, SSR markers can also indicate whether one or two copies of a particular chromosome segment are present. Several high-density maps containing SSR markers have been constructed for bread and durum wheat (Roder et al., 1998; Somers et al., 2004; Marone et al., 2012).

In the past decade, a number of other marker platforms have been developed, including mircroarray or gene chips that contain 1000s of unique probes spanning the entire wheat genome (Gupta et al., 2008). For large-scale screening, SSR markers have been largely replaced with single nucleotide polymorphism (SNP) markers covering the entire wheat genome with high density (Wang H. et al., 2014). In addition, the DArT (Diversity Array Technology) markers are based on a microarray platform that hybridizes the sample genome to identify the presence or absence of 1000s of unique fragements covering the whole genome (Jaccoud et al., 2001; Akbari et al., 2006). These DArT markers have been used to study genome inheritance and chromosome structure in pentaploid-derived wheat hybrid crosses (Eberhard et al., 2010; Martin et al., 2011). Recently, the DArT genotyping system has been improved by combining next-generation sequencing (NGS) with existing DArT markers to develop DArTseq^{TM}. DArTseq^{TM} markers have the potential to significantly increase the number of markers on each chromosome (Von Mark et al., 2013). This technique has been successfully applied to high-throughput screening of genetically diverse plant materials (Ren et al., 2015).

The majority of existing genotyping systems are based on dominant markers, which fail to differentiate between the presence of a single or two copies of a particular locus. Thus, it is impossible to detect incomplete or partial chromosomes in the presence of a complete homologous chromosome. Hence, the information generated through molecular markers alone is not sufficient to validate the allotetraploid or allohexaploid lines that show chromosome deletions, additions, or translocations. In such instances, it is essential to apply cytological techniques, such as GISH or MCFISH, which provide a more systematic approach for analyzing complex chromosomal complements (Eberhard et al., 2010).

CHALLENGES OF PRODUCING PENTAPLOID WHEAT HYBRIDS

Pentaploid hybrid production can be complex and requires careful consideration before any crosses are commenced. Common difficulties encountered and important points to

consider while developing pentaploid hybrids are summarized below.

Cross Direction (Hexaploid/Tetraploid or Tetraploid/Hexaploid)

To obtain the highest number of fertile F_1 progeny from an interspecific cross, it has been proposed that the higher ploidy level species should be used as the maternal parent (Kihara, 1982). In most studies of pentaploid hybrids to date, a hexaploid wheat has been used as the maternal parent (Mesfin et al., 1999; Lanning et al., 2008; Eberhard et al., 2010; Martin et al., 2011; Kalous et al., 2015). Crosses using the lower ploidy level species as the female generally have been less successful and can lead to poor seed set and subsequent low levels of seed germination and seedling establishment (Sharma and Gill, 1983). However, successful pentaploid hybrid crosses combining tetraploid wheat as the maternal parent and hexaploid wheat as the paternal parent have been reported (Wang et al., 2005).

Seedling Abnormailities

The F_1 seeds from inter-ploidy hybridisation between bread and durum wheat have relatively poor germination compared to seeds from crosses of parents of the same ploidy level (Kihara, 1982; Sharma and Gill, 1983) and can take several weeks to germinate. Complete failure of germination has been encountered in the reciprocal crosses between *T. monococcum* and *T. aestivum* (Kihara, 1982; Bhagyalakshmi et al., 2008). Unsurprisingly, a strong correlation between seed germination and seed morphology has been observed, with shriveled seeds showing poor germination compared to plump seeds (Kihara, 1982).

In interploidy crosses, normal seed development depends on the ploidy ratio of the maternal and paternal parent (Johnston et al., 1980; Carputo et al., 1999). Each species has been assigned a unique endosperm balance number (EBN). It is generally believed that the ploidy level of an embryo and its associated endosperm is critical for successful seed development (Ramsey and Schemske, 1998). Endosperm development is significant and has a major physiological and genetic relationship with the embryo of the newly formed allopolyploids. Johnston et al. (1980) explained the differences in EBN in an interspecific cross using tetraploid and diploid parent 4EBN(4x)/2EBN(2x) and its reciprocal cross. They indicated that progeny from the 4EBN/2EBN cross had a maternal:paternal ratio of endosperm of 4:1, while the reciprocal cross 2EBN/4EBN had a ratio of 1:1 which deviates from the normal 2:1 maternal:paternal ratio. EBN signifies the importance of endosperm development in inter ploidy crosses.

Even if F_1 pentaploid seeds germinate normally, chlorophyll abnormalities can develop, such as striato-virescence, delayed virescence, and albino expression (Tsunewaki, 2004). Furthermore, Tsunewaki (2004) showed that chlorophyll abnormalities were observed in the tetraploid wheat species with two duplicated recessive genes controlling the abnormalities. Hexaploid wheat lines carry wild-type homoeoalleles *Sv3*, *Dv3*, and *Abn3* for these abnormalities on chromosome 2D. Furthermore, abnormalities related to growth and development, such as stunted growth, grass clumping, and differences in flowering time, have been reported in interploidy wheat hybrids (Chen and Ni, 2006). While appropriate selection processes can rapidly remove these obvious abnormalities from subsequent pentaploid-derived generations, abnormalities with less obvious phenotypes may be harder to exclude.

Pollen Viability

Low pollen viability can also restrict seed set. The imbalanced chromosome number in the F_1 individuals impacts pollen development and subsequent fertilization (Kihara, 1982). The affected pollen grains do not germinate, the pollen tubes fail to reach the ovary, or the male and female gametes fail to fuse (Sharma and Gill, 1983). Pentaploid hybrids derived from a cross between *T. timopheevii* (AAGG) and *T. aestivum* produced completely sterile white anthers with infertile pollen (Bhagyalakshmi et al., 2008). The tissue that forms the pollen grain has a lower threshold for respiratory deficiency than do other plant tissues, which can lead to a loss of pollen viability that has been associated with the expression of novel mitochondrial peptides (Leon et al., 1998). Nucleotide rearrangement in certain genes, including the *atpa* locus, is considered to be the major cause of cytoplasmic male sterility in many crops (Chase, 1994, 2007; Heazlewood et al., 2003).

Apart from pollen viability, several other mutations, such as infertility, leaf striping, and severe growth impairments, could possibly arise from certain combinations of nuclear cytoplasm due to loss of mitochondrial genes (Newton and Coe, 1986). This evidence suggests that the mitochondrial genome may play a vital role in mediating the viability of pollen grains in many species, including interspecific wheat hybrids.

Progressive Hybrid Necrosis

Progressive hybrid necrosis can affect F_1 hybrids, resulting in prolonged chlorosis of plant leaf and sheath tissue. These symptoms lead to the premature death of leaves and tillers and eventually the whole plant in certain wheat hybrids (Caldwell and Compton, 1943; Hermsen, 1963a,b). Progressive necrosis is a lethal or semi-lethal condition that imposes a great barrier when trying to transfer desirable traits between species (Chu et al., 2006). This condition in F_1 hybrids is predominantly controlled by two complementary genes, *Ne1* and *Ne2*, located on the long arm of chromosome 5B and the short arm of chromosome 2B, respectively (Nishikawa et al., 1974). Both genes exist in bread and durum wheats (Tsunewaki, 1970, 1992, 2004). Recent genetic and mutational studies have found that the *Ne2* gene is closely related to the leaf rust resistance gene *Lr13* (Zhang et al., 2016). Knowledge of which bread and durum wheat genotypes carry alleles of the *Ne1* and *Ne2* genes will allow plant breeders to avoid the occurrence of progressive necrosis.

Nuclear-Cytoplasmic Interaction

Nuclear-cytoplasmic interaction (NCI) is the condition in which an interspecific hybrid possessing the nucleus of one parent interacts with cytoplasm inherited from the other parent (Simons et al., 2003). Pentaploid progeny with an unfavorable NCI exhibit

a wide range of phenotypes, such as maternally inherited male sterility, female infertility, late maturity, reduced vigor, pigment deficiencies, and altered morphology of cotyledons, leaves, and flowers (Monika et al., 2013).

The NCI in an interspecific hybrid is expressed by species cytoplasmic specific (*scs*) genes (Monika et al., 2013). In favorable interactions, these *scs* genes maintain the NCI and provide sufficient vigor and viability to the alloplasmic lines (Maan, 1992). The *scs* genes are located on chromosomes 1DL of *T. aestivum* (*scsae*) and 1AL of *T. timopheeviii* (*scsti*). *Triticum turgidum* spp. *durum* has a segment in chromosome 1A carrying *scsti* transferred from *T. timopheevii* (Simons et al., 2003). The *scs* gene plays a major role only when the nucleus of *T. aestivum* or *T. turgidum* spp. *durum* is combined with the cytoplasm of a wild species; otherwise, the *scs* gene stays unexpressed in normal cells with a compatible nucleus and cytoplasm (Monika et al., 2013).

Cytoplasmic organelles such as chloroplast and mitochondria are uniparentally inherited, mainly through the maternal lineage. Inheritance of paternal and novel copies of mitochondrial genes has been witnessed in a number of inter-ploidy wheat crosses. The mitochondrial heteroplasmy in inter-ploidy hybrids between wheat (6x) and rye (2x) (Laser et al., 1997), wheat (6x) and *Aegilops* sp. (2x,4x,6x) (Hattori et al., 2002), wheat (6x) and *T. timopheevi* (4x) (Kitagawa et al., 2003) and between barley (2x) and wheat (6x) (Aksyonova et al., 2005) have all been the subject of study. However, no attention has been given to hexaploid/tetraploid wheat crosses.

Fourty different SNP's have been identified between the hexaploid bread and tetraploid durum wheat mitochondrial genome. Five of these were present in known mitochondrial genes such as *rps1*, *rps2*, *cox3,* and *ccmFN* (Ogihara et al., 2005; Cui et al., 2009; Noyszewski et al., 2014). Developing SNP markers covering these identified genes would help to differentiate between bread and durum wheat mitochondrial genomes which might provide new insights regarding cytoplasmic inheritance in pentaploid wheat hybrids.

CONCLUSION AND FUTURE DIRECTIONS

Although, the value of genetic variability generated from hexaploid/tetraploid or tetraploid/hexaploid crosses is vast this technique has generally not been taken up in plant breeding programs. It is evident from research conducted to date, that pentaploid derived wheat lines would be valuable in commercial plant breeding programs that aim to improve fungal disease resistance, abiotic stress tolerance, quality parameters and agronomic characters. There are a number of other candidate traits, that could be potentially incorporated through pentaploid-derived wheat lines. One of these is nematode tolerance/resistance as durum wheats in general are more resistant to root leison nematode (*Pratylenchus thornei*) disease than bread wheats (Owen et al., 2010; Sheedy et al., 2012, 2015; Thompson et al., 2012). The transfer of some stress tolerant characteristics have been successful, but incorporating drought, cold, and heat tolerance characteristics using pentaploid-dervied wheat lines has not been studied in detail. There is a great potential to improve these traits using interspecific hybridisation as there is a wide variation for stress adaptive traits in the *Triticum* gene pool (Reynolds et al., 2009).

Although there are many barriers that restrict the production of pentaploid wheat hybrids, choice of parental genotype and using the higher ploidy level species as the maternal parent can improve the success rate. Improving the molecular and cytological techniques used to screen recombinant progenies will increase the efficiency of the selection process and help breeders in accelerating pentaploid production. Hence, interploidy hybridisation may be a promising tool for developing wheat genotypes that can cope with changing climate conditions. Therefore, it is essential to initiate further research to incorporate such traits from bread into durum wheat or durum into bread wheat through pentaploid wheat hybrids to assist the sustainable and increased global wheat production which will be required in the future.

AUTHOR CONTRIBUTIONS

SP conducted the literature survey and wrote the manuscript. PZ, RH, and MS improved the manuscript by comments and suggestions. AM initiated the project and contributed to manuscript.

ACKNOWLEDGMENTS

This study was partly funded by the Grains Research and Devlopment Corportation, Australia. Sriram Padmanaban acknowledges the support of an Australian Postgraduate Award. The authors would like to thank Dr. Kathleen L. Farquharson (Plant Editors) for her critical comments on this manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2017.00358/full#supplementary-material

REFERENCES

Akbari, M., Wenzl, P., Caig, V., Carling, J., Xia, L., Yang, S., et al. (2006). Diversity arrays technology (DArT) for high-throughput profiling of the hexaploid wheat genome. *Theor. Appl. Genet.* 113, 1409–1420. doi: 10.1007/s00122-006-0365-4

Aksyonova, E., Sinyavskaya, M., Danilenko, N., Pershina, L., Nakamura, C., and Davydenko, O. (2005). Heteroplasmy and paternally oriented shift of the organellar DNA composition in barley–wheat hybrids during backcrosses with wheat parents. *Genome* 48, 761–769. doi: 10.1139/g05-049

Bai, G., and Shaner, G. (2004). Management and resistance in wheat and barley to fusarium head blight. *Annu. Rev. Phytopathol.* 42, 135–161. doi: 10.1146/annurev.phyto.42.040803.140340

Bhagyalakshmi, K., Vinod, K. K., Kumar, M., Arumugachamy, S., Prabhakaran, A., and Raveendran, T. S. (2008). Interspecific hybrids from wild x cultivated *Triticum* crosses-a study on the cytological behaviour and molecular relations. *J. Crop Sci. Biotechnol.* 11, 257–262.

Bottalico, A., and Perrone, G. (2002). Toxigenic *Fusarium* species and mycotoxins associated with head blight in small-grain cereals in Europe. *Eur. J. Plant Pathol.* 108, 611–624. doi: 10.1023/A:1020635214971

Bovill, W., Ma, W., Ritter, K., Collard, B., Davis, M., Wildermuth, G., et al. (2006). Identification of novel QTL for resistance to crown rot in the doubled haploid wheat population 'W21MMT70' × 'Mendos'. *Plant Breed.* 125, 538–543. doi: 10.1111/j.1439-0523.2006.01251.x

Bovill, W. D., Horne, M., Herde, D., Davis, M., Wildermuth, G. B., and Sutherland, M. (2010). Pyramiding QTL increases seedling resistance to crown rot (*Fusarium pseudograminearum*) of wheat (*Triticum aestivum*). *Theor. Appl. Genet.* 121, 127–136. doi: 10.1007/s00122-010-1296-7

Caldwell, R. M., and Compton, L. (1943). Complementary lethal genes in wheat causing a progressive lethal necrosis of seedlings. *J. Hered.* 34, 67–70. doi: 10.1093/oxfordjournals.jhered.a105248

Carputo, D., Monti, L., Werner, J. E., and Frusciante, L. (1999). Uses and usefulness of endosperm balance number. *Theor. Appl. Genet.* 98, 478–484. doi: 10.1007/s001220051095

Chase, C. D. (1994). Expression of CMS-unique and flanking mitochondrial DNA sequences in *Phaseolus vulgaris* L. *Curr. Genet.* 25, 245–251. doi: 10.1007/BF00357169

Chase, C. D. (2007). Cytoplasmic male sterility: a window to the world of plant mitochondrial–nuclear interactions. *Trends Genet.* 23, 81–90. doi: 10.1016/j.tig.2006.12.004

Chen, X. (2013). Review article: high-temperature adult-plant resistance, key for sustainable control of stripe rust. *Am. J. Plant Sci.* 4, 608–627. doi: 10.4236/ajps.2013.43080

Chen, Z. J., and Ni, Z. (2006). Mechanisms of genomic rearrangements and gene expression changes in plant polyploids. *Bioessays* 28, 240–252. doi: 10.1002/bies.20374

Chu, C.-G., Faris, J., Friesen, T., and Xu, S. (2006). Molecular mapping of hybrid necrosis genes *Ne1* and *Ne2* in hexaploid wheat using microsatellite markers. *Theor. Appl. Genet.* 112, 1374–1381. doi: 10.1007/s00122-006-0239-9

Clear, R., and Patrick, S. (1990). *Fusarium* species isolated from wheat samples containing tombstone (scab) kernels from Ontario, Manitoba, and Saskatchewan. *Can. J. Plant Sci.* 70, 1057–1069. doi: 10.4141/cjps90-128

Colmer, T. D., Flowers, T. J., and Munns, R. (2006). Use of wild relatives to improve salt tolerance in wheat. *J. Exp. Bot.* 57, 1059–1078. doi: 10.1093/jxb/erj124

Cui, P., Liu, H., Lin, Q., Ding, F., Zhuo, G., Hu, S., et al. (2009). A complete mitochondrial genome of wheat (*Triticum aestivum* cv. Chinese Yumai), and fast evolving mitochondrial genes in higher plants. *J. Genet.* 88, 299–307. doi: 10.1007/s12041-009-0043-9

Eberhard, F. S., Zhang, P., Lehmensiek, A., Hare, R. A., Simpfendorfer, S., and Sutherland, M. W. (2010). Chromosome composition of an F2 *Triticum aestivum* X *T.turgidum* spp. *durum* cross analysed by DArT markers and MCFISH. *Crop Pasture Sci.* 61, 619–624. doi: 10.1071/CP10131

Friebe, B., Jiang, J., Raupp, W., and Gill, B. (1993). "Molecular cytogenetic analysis of radiation-induced alien genetic transfers in wheat," in *Proceedings of the Eighth International Wheat Genetics Symposium*, Beijing, 20–25.

Friebe, B., Jiang, J., Raupp, W., Mcintosh, R., and Gill, B. (1996). Characterization of wheat-alien translocations conferring resistance to diseases and pests: current status. *Euphytica* 91, 59–87. doi: 10.1007/BF00035277

Galili, G., and Feldman, M. (1984). Intergenomic suppression of endosperm protein genes in common wheat. *Can. J. Genet. Cytol.* 26, 651–656. doi: 10.1007/BF00281853

Gall, J. G., and Pardue, M. L. (1969). Formation and detection of RNA-DNA hybrid molecules in cytological preparations. *Proc. Natl. Acad. Sci. U.S.A.* 63, 378–383. doi: 10.1073/pnas.63.2.378

Gilbert, J., Procunier, J., and Aung, T. (2000). Influence of the D genome in conferring resistance to fusarium head blight in spring wheat. *Euphytica* 114, 181–186. doi: 10.1023/A:1004065620127

Gupta, P., Rustgi, S., and Mir, R. (2008). Array-based high-throughput DNA markers for crop improvement. *Heredity* 101, 5–18. doi: 10.1038/hdy.2008.35

Han, C., Ryan, P. R., Yan, Z., and Delhaize, E. (2014). Introgression of a 4D chromosomal fragment into durum wheat confers aluminium tolerance. *Ann. Bot.* 114, 135–144. doi: 10.1093/aob/mcu070

Han, C., Zhang, P., Ryan, P. R., Rathjen, T. M., Yan, Z., and Delhaize, E. (2016). Introgression of genes from bread wheat enhances the aluminium tolerance of durum wheat. *Theor. Appl. Genet.* 129, 729–739. doi: 10.1007/s00122-015-2661-3

Hattori, N., Kitagawa, K., Takumi, S., and Nakamura, C. (2002). Mitochondrial DNA heteroplasmy in wheat, *Aegilops* and their nucleus-cytoplasm hybrids. *Genetics* 160, 1619–1630.

Heazlewood, J., Whelan, J., and Millar, A. (2003). The products of the mitochondrial *orf25* and *orfB* genes are FO components in the plant F1FO ATP synthase. *FEBS Lett.* 540, 201–205. doi: 10.1016/S0014-5793(03)00264-3

Hermsen, J. T. (1963a). The genetic basis of hybrid necrosis in wheat. *Genetica* 33, 245–287. doi: 10.1007/BF01725765

Hermsen, J. T. (1963b). Sources and distribution of the complementary genes for hybrid necrosis in wheat. *Euphytica* 12, 147–160. doi: 10.1007/BF00022351

Jaccoud, D., Peng, K., Feinstein, D., and Kilian, A. (2001). Diversity arrays: a solid state technology for sequence information independent genotyping. *Nucleic Acids Res.* 29:e25. doi: 10.1093/nar/29.4.e25

Jiang, J., Friebe, B., and Gill, B. S. (1993). Recent advances in alien gene transfer in wheat. *Euphytica* 73, 199–212. doi: 10.1038/hdy.2012.116

Johnston, S., Den Nijs, T., Peloquin, S., and Hanneman, R. Jr. (1980). The significance of genic balance to endosperm development in interspecific crosses. *Theor. Appl. Genet.* 57, 5–9. doi: 10.1007/BF00276002

Joppa, L., and Williams, N. (1988). Langdon durum disomic substitution lines and aneuploid analysis in tetraploid wheat. *Genome* 30, 222–228. doi: 10.1139/g88-038

Kalous, J., Martin, J., Sherman, J., Heo, H.-Y., Blake, N., Lanning, S., et al. (2015). Impact of the D genome and quantitative trait loci on quantitative traits in a spring durum by spring bread wheat cross. *Theor. Appl. Genet.* 128, 1799–1811. doi: 10.1007/s00122-015-2548-3

Kihara, H. (1924). Cytologische und genetische Studien bei wichtigen Getreidearten mit besondere Rüchsicht auf das Verhalten der Chromosomen und die Sterilität in den Bastarden. *Mem. Coll. Sci. Kyoto Imp. Univ. B* 1, 1–200.

Kihara, H. (1982). *Wheat Studies Retrospect and Prospects*. Tokyo: Kodansha Ltd.

Kitagawa, K., Takumi, S., and Nakamura, C. (2003). Selective transcription and post-transcriptional processing of the heteroplasmic mitochondrial *orf156* copies in the nucleus-cytoplasm hybrids of wheat. *Plant Mol. Biol.* 53, 609–619. doi: 10.1023/B:PLAN.0000019075.31353.77

Komuro, S., Endo, R., Shikata, K., and Kato, A. (2013). Genomic and chromosomal distribution patterns of various repeated DNA sequences in wheat revealed by a fluorescence in situ hybridization procedure. *Genome* 56, 131–137. doi: 10.1139/gen-2013-0003

Koo, D.-H., Sehgal, S. K., Friebe, B., and Gill, B. S. (2015). Structure and stability of telocentric chromosomes in wheat. *PLoS ONE* 10:e0137747. doi: 10.1371/journal.pone.0137747

Lanning, S. P., Blake, N. K., Sherman, J. D., and Talbert, L. E. (2008). Variable production of tetraploid and hexaploid progeny lines from spring wheat by durum wheat crosses. *Crop Sci.* 48, 199–202. doi: 10.2135/cropsci2007.06.0334

Laser, B., Mohr, S., Odenbach, W., Oettler, G., and Kück, U. (1997). Parental and novel copies of the mitochondrial *orf25* gene in the hybrid crop-plant triticale: predominant transcriptional expression of the maternal gene copy. *Curr. Genet.* 32, 337–347. doi: 10.1007/s002940050286

Le, H. T., Armstrong, K., and Miki, B. (1989). Detection of rye DNA in wheat-rye hybrids and wheat translocation stocks using total genomic DNA as a probe. *Plant Mol. Biol. Rep.* 7, 150–158. doi: 10.1007/BF02669631

Leon, P., Arroyo, A., and Mackenzie, S. (1998). Nuclear control of plastid and mitochondrial development in higher plants. *Annu. Rev. Plant Biol.* 49, 453–480. doi: 10.1146/annurev.arplant.49.1.453

Lindsay, M. P., Lagudah, E. S., Hare, R. A., and Munns, R. (2004). A locus for sodium exclusion (*Nax1*), a trait for salt tolerance, mapped in durum wheat. *Funct. Plant Biol.* 31, 1105–1114. doi: 10.1071/FP04111

Liu, C., and Ogbonnaya, F. C. (2015). Resistance to Fusarium crown rot in wheat and barley: a review. *Plant Breed.* 134, 365–372. doi: 10.1111/mpp.12011

Liu, C.-Y., Shepherd, K. W., and Rathjen, J. R. (1996). Improvement of durum wheat pastamaking and breadmaking qualities. *Cereal Chem.* 73, 155–166.

Lopes, M., Reynolds, M., Draye, X., Foulkes, J., Hawkesford, M., and Murchie, E. (2010). Dissecting drought adaptation into its phenotypic and genetic components in wheat. *Asp. Appl. Biol.* 105, 7–11.

Maan, S. (1992). A gene for embryo-endosperm compatibility and seed viability in alloplasmic *Triticum turgidum*. *Genome* 35, 772–779. doi: 10.1139/g92-118

Marone, D., Laido, G., Gadaleta, A., Colasuonno, P., Ficco, D. B., Giancaspro, A., et al. (2012). A high-density consensus map of A and B wheat genomes. *Theor. Appl. Genet.* 125, 1619–1638. doi: 10.1007/s00122-012-1939-y

Martin, A., Bovill, W., Percy, C., Herde, D., Fletcher, S., Kelly, A., et al. (2015). Markers for seedling and adult plant crown rot resistance in four partially resistant bread wheat sources. *Theor. Appl. Genet.* 128, 377–385. doi: 10.1007/s00122-014-2437-1

Martin, A., Simpfendorfer, S., Hare, R., and Sutherland, M. (2013). Introgression of hexaploid sources of crown rot resistance into durum wheat. *Euphytica* 192, 463–470. doi: 10.1007/s10681-013-0890-6

Martin, A., Simpfendorfer, S., Hare, R. A., Eberhard, F. S., and Sutherland, M. W. (2011). Retention of D genome chromosomes in pentaploid wheat crosses. *Heredity* 107, 315–319. doi: 10.1038/hdy.2011.17

Matny, O. N. (2015). *Fusarium* head blight and crown rot on wheat & barley: losses and health risks. *Adv. Plants Agric. Res.* 2:39. doi: 10.15406/apar.2015.02.00039

Matsuoka, Y. (2011). Evolution of polyploid triticum wheats under cultivation: the role of domestication, natural hybridization and allopolyploid speciation in their diversification. *Plant Cell Physiol.* 52, 750–764. doi: 10.1093/pcp/pcr018

Mesfin, A., Frohberg, R. C., and Anderson, J. A. (1999). RFLP markers associated with high grain protein from *Triticum turgidum* L. var. *dicoccoides* introgressed into hard red spring wheat. *Crop Sci.* 39, 508–513. doi: 10.2135/cropsci1999.0011183X003900020035x

Monika, K., Jimenez, M. D., Bassi, F. M., Ghavami, F., Simons, K., Dizon, R., et al. (2013). A radiation hybrid map of chromosome 1D reveals synteny conservation at a wheat speciation locus. *Funct. Integr. Genomics* 13, 19–32. doi: 10.1007/s10142-013-0318-3

Mukai, Y., Nakahara, Y., and Yamamoto, M. (1993). Simultaneous discrimination of the three genomes in hexaploid wheat by multicolor fluorescence in situ hybridization using total genomic and highly repeated DNA probes. *Genome* 36, 489–494. doi: 10.1139/g93-067

Munns, R., Hare, R., James, R., and Rebetzke, G. (1999). Genetic variation for improving the salt tolerance of durum wheat. *Crop Pasture Sci.* 51, 69–74. doi: 10.1071/AR99057

Navashin, M. (1934). Chromosome alterations caused by hybridization and their bearing upon certain general genetic problems. *Cytologia* 5, 169–203. doi: 10.1508/cytologia.5.169

Newton, K. J., and Coe, E. (1986). Mitochondrial DNA changes in abnormal growth (nonchromosomal stripe) mutants of maize. *Proc. Natl. Acad. Sci. U.S.A.* 83, 7363–7366. doi: 10.1073/pnas.83.19.7363

Nishikawa, K., Mori, T., Takami, N., and Furuta, Y. (1974). Mapping of progressive necrosis genes, *Ne1* and *Ne2* of common wheat by the telocentric method. *Jpn. J. Breed.* 24, 277–281. doi: 10.1270/jsbbs1951.24.277

Novaro, P., D'egidio, M., Mariani, B., and Nardi, S. (1993). Combined effect of protein content and high-temperature drying systems on pasta cooking quality. *Cereal Chem.* 70, 716–716.

Noyszewski, A. K., Ghavami, F., Alnemer, L. M., Soltani, A., Gu, Y. Q., Huo, N., et al. (2014). Accelerated evolution of the mitochondrial genome in an alloplasmic line of durum wheat. *BMC Genomics* 15:67. doi: 10.1186/1471-2164-15-67

Ogihara, Y., Yamazaki, Y., Murai, K., Kanno, A., Terachi, T., Shiina, T., et al. (2005). Structural dynamics of cereal mitochondrial genomes as revealed by complete nucleotide sequencing of the wheat mitochondrial genome. *Nucleic Acids Res.* 33, 6235–6250. doi: 10.1093/nar/gki925

Owen, K., Clewett, T., and Thompson, J. (2010). Pre-cropping with canola decreased *Pratylenchus thornei* populations, arbuscular mycorrhizal fungi, and yield of wheat. *Crop Pasture Sci.* 61, 399–410. doi: 10.1071/CP09345

Palumbo, M., Spina, A., and Boggini, G. (2000). "Agronomic and bread-making characteristics of durum wheat genotypes deriving from interspecific hybridisation with bread wheat," in *Durum Wheat Improvement in the Mediterranean Region: New Challenges*, eds J. L. Araus, N. Di Fonzo, M. Nachit, and C. Royo (Zaragoza: CIHEAM), 515–518.

Pikaard, C. S. (1999). Nucleolar dominance and silencing of transcription. *Trends Plant Sci.* 4, 478–483. doi: 10.1016/S1360-1385(99)01501-0

Ramsey, J., and Schemske, D. W. (1998). Pathways, mechanisms, and rates of polyploid formation in flowering plants. *Annu. Rev. Ecol. Syst.* 29, 467–501. doi: 10.1146/annurev.ecolsys.29.1.467

Rayburn, A. L., and Gill, B. S. (1986). Isolation of a D-genome specific repeated DNA sequence from *Aegilops squarrosa*. *Plant Mol. Biol. Rep.* 4, 102–109. doi: 10.1007/BF02732107

Reader, S., and Miller, T. (1991). The introduction into bread wheat of a major gene for resistance to powdery mildew from wild emmer wheat. *Euphytica* 53, 57–60. doi: 10.1007/BF00032033

Ren, R., Ray, R., Li, P., Xu, J., Zhang, M., Liu, G., et al. (2015). Construction of a high-density DArTseq SNP-based genetic map and identification of genomic regions with segregation distortion in a genetic population derived from a cross between feral and cultivated-type watermelon. *Mol. Genet. Genomics* 290, 1457–1470. doi: 10.1007/s00438-015-0997-7

Reynolds, M., Manes, Y., Izanloo, A., and Langridge, P. (2009). Phenotyping approaches for physiological breeding and gene discovery in wheat. *Ann. Appl. Biol.* 155, 309–320. doi: 10.1111/j.1744-7348.2009.00351.x

Roder, M. S., Korzun, V., Wendehake, K., Plaschke, J., Tixier, M.-H. N., Leroy, P., et al. (1998). A microsatellite map of wheat. *Genetics* 149, 2007–2023.

Rong, J., Millet, E., Manisterski, J., and Feldman, M. (2000). A new powdery mildew resistance gene: introgression from wild emmer into common wheat and RFLP-based mapping. *Euphytica* 115, 121–126. doi: 10.1023/A:1003950431049

Schubert, I., Fransz, P. F., Fuchs, J., and De Jong, J. H. (2001). "Chromosome painting in plants," in *Chromosome Painting*, eds A. K. Sharma and A. Sharma (Dordrecht: Springer), 57–69. doi: 10.1007/978-94-010-0330-8_7

Schwarzacher, T., Anamthawat-Jonsson, K., Harrison, G., Islam, A., Jia, J., King, I., et al. (1992). Genomic in situ hybridization to identify alien chromosomes and chromosome segments in wheat. *Theor. Appl. Genet.* 84, 778–786. doi: 10.1007/BF00227384

Schwarzacher, T., Leitch, A., Bennett, M., and Heslop-Harrison, J. (1989). In situ localization of parental genomes in a wide hybrid. *Ann. Bot.* 64, 315–324. doi: 10.1093/oxfordjournals.aob.a087847

Sharma, H. C., and Gill, B. S. (1983). Current status of wide hybridization in wheat. *Euphytica* 32, 17–31. doi: 10.1007/BF00036860

Sheedy, J., Mckay, A., Lewis, J., Vanstone, V., Fletcher, S., Kelly, A., et al. (2015). Cereal cultivars can be ranked consistently for resistance to root-lesion nematodes (*Pratylenchus thornei* & *P. neglectus*) using diverse procedures. *Australas. Plant Pathol.* 44, 175–182. doi: 10.1007/s13313-014-0333-4

Sheedy, J., Thompson, J., and Kelly, A. (2012). Diploid and tetraploid progenitors of wheat are valuable sources of resistance to the root lesion nematode *Pratylenchus thornei*. *Euphytica* 186, 377–391. doi: 10.1007/s10681-011-0617-5

Shepherd, K. (1988). "Genetics of wheat endosperm proteins-in retrospect and prospect," in *Proceedings of the Seventh International Wheat Genetics Symposium*, eds T. E. Miller and R. M. D. Koebner (Oxford: Oxford University Press), 919–931.

Shewry, P., Halford, N., and Tatham, A. (1992). High molecular weight subunits of wheat glutenin. *J. Cereal Sci.* 15, 105–120. doi: 10.1016/S0733-5210(09)80062-3

Silva, G., and Souza, M. (2013). Genomic in situ hybridization in plants. *Genet. Mol. Res.* 12, 2953–2965. doi: 10.4238/2013.August.12.11

Simons, K. J., Gehlhar, S. B., Maan, S. S., and Kianian, S. F. (2003). Detailed mapping of the species cytoplasm-specific (*scs*) gene in durum wheat. *Genetics* 165, 2129–2136.

Sissons, M. (2008). Role of durum wheat composition on the quality of pasta and bread. *Food* 2, 75–90. doi: 10.1371/journal.pone.0050057

Sissons, M. (2012). *Durum Wheat Chemistry and Technology*. London: Academic Press.

Smiley, R. W., Gourlie, J. A., Easley, S. A., Patterson, L.-M., and Whittaker, R. G. (2005). Crop damage estimates for crown rot of wheat and barley in the Pacific Northwest. *Plant Dis.* 89, 595–604. doi: 10.1094/PD-89-0595

Somers, D. J., Isaac, P., and Edwards, K. (2004). A high-density microsatellite consensus map for bread wheat (*Triticum aestivum* L.). *Theor. Appl. Genet.* 109, 1105–1114. doi: 10.1007/s00122-004-1740-7

Thompson, J. P., Zwart, R. S., and Butler, D. (2012). Inheritance of resistance to root-lesion nematodes (*Pratylenchus thornei* and *P. neglectus*) in five doubled-haploid populations of wheat. *Euphytica* 188, 209–219. doi: 10.1007/s10681-012-0689-x

Troccoli, A., Borrelli, G., De Vita, P., Fares, C., and Di Fonzo, N. (2000). Mini review: durum wheat quality: a multidisciplinary concept. *J. Cereal Sci.* 32, 99–113. doi: 10.1006/jcrs.2000.0322

Tsunewaki, K. (1970). Necrosis and chlorosis genes in common wheat and its ancestral species. *Seiken Ziho* 22, 67–75.

Tsunewaki, K. (1992). Aneuploid analyses of hybrid necrosis and hybrid chlorosis in tetraploid wheats using the D genome chromosome substitution lines of durum wheat. *Genome* 35, 594–601. doi: 10.1139/g92-089

Tsunewaki, K. (2004). Aneuploid analyses of three chlorophyll abnormalities in Emmer wheat. *Genes Genet. Syst.* 79, 95–104. doi: 10.1266/ggs.79.95

Von Mark, V. C., Kilian, A., and Dierig, D. A. (2013). Development of DArT marker platforms and genetic diversity assessment of the US collection of the new oilseed crop lesquerella and related species. *PLoS ONE* 8:e64062. doi: 10.1371/journal.pone.0064062

Wang, H., Jiang, J., Chen, S., Qi, X., Fang, W., Guan, Z., et al. (2014). Rapid genetic and epigenetic alterations under intergeneric genomic shock in newly synthesized *Chrysanthemum morifolium* × *Leucanthemum paludosum* hybrids (Asteraceae). *Genome Biol. Evol.* 6, 247–259. doi: 10.1093/gbe/evu008

Wang, H., Liu, D., Yan, Z., Wei, Y., and Zheng, Y. (2005). Cytological characteristics of F2 hybrids between *Triticum aestivum* L. and *T. durum* Desf. with reference to wheat breeding. *J. Appl. Genet.* 46, 365–369.

Wang, S., Wong, D., Forrest, K., Allen, A., Chao, S., Huang, B. E., et al. (2014). Characterization of polyploid wheat genomic diversity using a high-density 90 000 single nucleotide polymorphism array. *Plant Biotechnol. J.* 12, 787–796. doi: 10.1111/pbi.12183

Wildermuth, G., Mcnamara, R., and Quick, J. (2001). Crown depth and susceptibility to crown rot in wheat. *Euphytica* 122, 397–405. doi: 10.1023/A:1012947516161

Xu, L., Wang, M., Cheng, P., Kang, Z., Hulbert, S., and Chen, X. (2013). Molecular mapping of *Yr53*, a new gene for stripe rust resistance in durum wheat accession PI 480148 and its transfer to common wheat. *Theor. Appl. Genet.* 126, 523–533. doi: 10.1007/s00122-012-1998-0

Zhang, P., Friebe, B., Gill, B., and Park, R. (2007). Cytogenetics in the age of molecular genetics. *Crop Pasture Sci.* 58, 498–506. doi: 10.1071/AR07054

Zhang, P., Hiebert, C. W., Mcintosh, R. A., Mccallum, B. D., Thomas, J. B., Hoxha, S., et al. (2016). The relationship of leaf rust resistance gene *Lr13* and hybrid necrosis gene *Ne2m* on wheat chromosome 2BS. *Theor. Appl. Genet.* 129, 485–493. doi: 10.1007/s00122-015-2642-6

Zhang, P., Li, W., Friebe, B., and Gill, B. S. (2004). Simultaneous painting of three genomes in hexaploid wheat by BAC-FISH. *Genome* 47, 979–987. doi: 10.1139/g04-042

Zhang, W., Chao, S., Manthey, F., Chicaiza, O., Brevis, J. C., Echenique, V., et al. (2008). QTL analysis of pasta quality using a composite microsatellite and SNP map of durum wheat. *Theor. Appl. Genet.* 117, 1361–1377. doi: 10.1007/s00122-008-0869-1

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Supplementary Table-1 Pentaploid wheat hybrids: challenges, protocols, and applications
Sriram Padmanaban, Peng Zhang, Ray A. Hare, Mark W. Sutherland, and Anke Martin

Study	Female	Male	Generation	Number of lines	Group 1	Group 2
Kihara	Spelta 6X	polonicum 4X	F2	433	416	17
Wang et al., 2005	As295 4X	CN16 6X	F5	55	52	3
Lanning et al., 2008	Choteau 6X	Avonlea 4X	F5	66	37	29
Lanning et al., 2008	MT9565 6X	Avonlea 4X	F5	62	51	11
Lanning et al., 2008	Chinese Spring 6X	Avonlea 4X	F5	23	6	17
Lanning etal., 2008	Hank 6X	Avonlea 4X	F5	12	11	1
Lanning et al., 2008	Len 6X	Avonlea 4X	F5	8	7	1
Lanning et al., 2008	Ernest 6X	Avonlea 4X	F5	10	6	4
Lanning et al., 2008	Bob white 6X	Monroe 4X	F5	1	0	1
Ebarhard et al., 2010	2-49 6X	Bellaroi 4X	F2	83	47	36
Ebarhard et al., 2010	2-49 6X	Bellaroi 4X	F2	26	24	2
Martin et al., 2011	2-49 6X	Bellaroi 4X	F3	59	48	11
Martin et al., 2011	2-49 6X	Bellaroi 4X	F7	16	12	4
Martin et al., 2011	2-49 6X	950329 4X	F6	43	32	11
Martin et al., 2011	Sunco 6X	Bellaroi 4X	F2	34	32	2
Martin etal., 2011	Sunco 6X	230604 4x	F2	29	29	0
Martin et al., 2011	CPI133814 6X	Bellaroi 4X	F3	38	38	0
Martin et al., 2011	CPI133814 6X	Bellaroi 4X	F7	12	12	0
Kalous et al., 2015	Choteau 6X	Mountrail 4X	F8	205	88	117
Kalous et al., 2015	Choteau 6X	Avonlea 4X	F8	15	2	13
Kalous et al., 2015	Choteau 6X	Monroe 4X	F8	14	9	5
Kalous et al., 2015	MT9565 6X	Avonlea 4X	F8	13	11	2
Kalous et al., 2015	MT9565 6X	Monroe 4X	F8	17	14	3
Kalous et al., 2015	MT9565 6X	Mountrail 4X	F8	14	9	5
Kalous et al., 2015	Bob white 6X	Monroe 4X	F8	4	2	2
Kalous et al., 2015	Chinese Spring 6X	Avonlea 4X	F8	4	0	4
Kalous et al., 2015	Chinese Spring 6X	Monroe 4X	F8	4	1	3
Kalous et al., 2015	Chinese Spring 6X	Mountrail 4X	F8	8	0	8
Kalous et al., 2015	Ernest 6X	Avonlea 4X	F8	13	7	6
Kalous et al., 2015	Ernest 6X	Monroe 4X	F8	10	8	2
Kalous et al., 2015	Hank 6X	Avonlea 4X	F8	8	5	3
Kalous et al., 2015	Hank 6X	Monroe 4X	F8	5	2	3
Kalous et al., 2015	Hank 6X	Mountrail 4X	F8	10	9	1
Kalous et al., 2015	Len 6X	Avonlea 4X	F8	5	5	0
Kalous et al., 2015	Len 6X	Monroe 4X	F8	2	2	0
Kalous et al., 2015	Len 6X	Mountrail 4X	F8	5	5	0
Kalous et al., 2015	MCNeal 6X	Monroe 4X	F8	1	1	0

Chapter 3

A cytological and molecular analysis of D-genome chromosome retention in the F_2 to F_6 generation of hexaploid x tetraploid wheat crosses

This study examined how the retention of D-genome chromosomes differs in various hexaploid/tetraploid wheat crosses over multiple generations using both molecular and cytological approaches. Different hexaploid/tetraploid crosses were developed having the same hexaploid maternal parent but different tetraploid paternal parents and *vice-versa*. Crosses were also made to study any effect of the time of crossing on the retention pattern of individual D chromosomes. These were examined in the F_2 generation. D-genome stability was also examined in one selected cross, across multiple generations up to the F_6 generation. The content of this chapter is published in the journal "Crop and Pasture Science" and details are below.

Padmanaban S, Zhang P, Sutherland MW, Knight NL and Martin A (2018) "A cytological and Molecular analysis of D-genome chromosome retention in the F_2 - F_6 generations of hexaploid/ tetraploid wheat crosses". **Crop and Pasture Science 2018, 69, 121-130.**

Note: Supplementary data associated with this chapter are attached along with the article.

CSIRO PUBLISHING
Crop & Pasture Science, 2018, **69**, 121–130
https://doi.org/10.1071/CP17240

A cytological and molecular analysis of D-genome chromosome retention following F_2–F_6 generations of hexaploid × tetraploid wheat crosses

Sriram Padmanaban[A], *Peng Zhang*[B], *Mark W. Sutherland*[A], *Noel L. Knight*[A,C], *and Anke Martin*[A,D]

[A]Centre for Crop Health, University of Southern Queensland, Toowoomba, Qld 4350, Australia.
[B]Plant Breeding Institute, The University of Sydney, Cobbitty, NSW 2570, Australia.
[C]Present address: School of Integrative Plant Science, Plant Pathology and Plant–Microbe Biology Section, Cornell University, Geneva, NY 14456, USA.
[D]Corresponding author. Email: anke.martin@usq.edu.au

Abstract. Both hexaploid bread wheat (AABBDD) (*Triticum aestivum* L.) and tetraploid durum wheat (AABB) (*T. turgidum* spp. *durum*) are highly significant global food crops. Crossing these two wheats with different ploidy levels results in pentaploid (AABBD) F_1 lines. This study investigated the differences in the retention of D chromosomes between different hexaploid × tetraploid crosses in subsequent generations by using molecular and cytological techniques. Significant differences ($P < 0.05$) were observed in the retention of D chromosomes in the F_2 generation depending on the parents of the original cross. One of the crosses, 2WE25 × 950329, retained at least one copy of each D chromosome in 48% of its F_2 lines. For this cross, the retention or elimination of D chromosomes was determined through several subsequent self-fertilised generations. Cytological analysis indicated that D chromosomes were still being eliminated at the F_5 generation, suggesting that in some hexaploid × tetraploid crosses, D chromosomes are unstable for many generations. This study provides information on the variation in D chromosome retention in different hexaploid × tetraploid wheat crosses and suggests efficient strategies for utilising D genome retention or elimination to improve bread and durum wheat, respectively.

Additional keywords: DArTseq, FISH, GISH, interploidy crosses.

Received 7 July 2017, accepted 26 November 2017, published online 5 February 2018

Introduction

The potential advantages of producing interspecific wheat hybrids by using different ploidy levels has been documented (Kihara 1982; Eberhard *et al*. 2010; Martin *et al*. 2011; Xu *et al*. 2013; Padmanaban *et al*. 2017*a*, 2017*b*). For example, hexaploid bread wheat (*Triticum aestivum* L.) has several desirable traits such as partial resistance to Fusarium crown rot (Martin *et al*. 2013) and Fusarium head blight (Gilbert *et al*. 2000), tolerance to salinity (Munns *et al*. 2000), and grain-quality attributes for improved bread making (Kalous *et al*. 2015). These traits can be incorporated into tetraploid durum wheat (*T. turgidum* spp. *durum*) by using interspecific crosses. Similarly, durum wheat shows enhanced tolerance to nematodes (Sheedy *et al*. 2015), together with desirable seed size and yield characteristics (Kalous *et al*. 2015), which could be transferred to bread wheat. Therefore, the development of efficient protocols for crossing hexaploid wheat and tetraploid wheat, together with appropriate strategies for selecting desirable lines from subsequent generations, will aid in the genetic improvement of these two closely related species.

For production of interspecific wheat hybrids, the higher ploidy-level species should be used as the maternal parent because this will result in a greater number of fertile F_1 progeny (Kihara 1982). Crosses in which the lower ploidy level species has been used as the female generally have been less successful and can lead to poor seedset and subsequent low levels of seed germination and seedling establishment (Sharma and Gill 1983; Padmanaban *et al*. 2017*a*, 2017*b*). Hybridisation between hexaploid wheat (AABBDD $2n = 6x = 42$) and tetraploid wheat (AABB $2n = 4x = 28$) results in interploidy F_1 progeny, henceforth called pentaploid hybrids, which have the genome constitution of AABBD ($2n = 5x = 35$) (Kihara 1924). Successful reciprocal crosses between tetraploid and hexaploid wheat were developed and studied by Wang *et al*. (2005) and Padmanaban *et al*. (2017*a*). Because of the pentaploid composition of this F_1 generation, subsequent derived generations undergo irregular chromosome pairing in the D genome. Thus, F_2 lines derived from pentaploid wheat crosses display various chromosome numbers ranging from $2n = 28$ to $2n = 42$, based on the retained number of copies

(0, 1 or 2) of each of the seven unique D-genome chromosomes. Several studies of pentaploid-derived generations have reported differences in the retention of D-genome chromosomes while identifying favourable allelic recombinations in the stable A and B genomes (Gilbert *et al*. 2000; Wang *et al*. 2005; Lanning *et al*. 2008; Eberhard *et al*. 2010).

Martin *et al*. (2011) reported that particular parental combinations played a key role in determining the retention of D chromosomes in the successive generations of hexaploid–tetraploid wheat crosses. This study indicated that hexaploid–tetraploid wheat crosses CPI133814/Bellaroi and Sunco/230604 may be highly suitable for durum wheat improvement because the D chromosomes were predominantly eliminated in the F_3 and F_2 generation, respectively. There also appears to be a strong correlation between the retention of D chromosomes and the inheritance of the A and B genomes. Lines that retained a large number of D chromosomes also inherited a higher proportion of the A and B genomes from bread wheat (Martin *et al.* 2011; Padmanaban *et al.* 2017*b*).

Microsatellite, Diversity Arrays Technology (DArT), and single nucleotide polymorphism (SNP) marker approaches have been widely used to assess the chromosomal composition of recombinant progeny of hexaploid–tetraploid wheat crosses (Martin *et al*. 2011; King *et al*. 2013; Kalous *et al*. 2015; Ren *et al*. 2015). Although molecular markers can be used to identify which unique D chromosomes are present, the number of copies of each D chromosome retained cannot be determined. Thus, cytological approaches play a significant role in identifying the copy number of D chromosomes retained in hexaploid–tetraploid progeny. Fluorescence *in situ* hybridisation (FISH) and genomic *in situ* hybridisation (GISH) techniques are used to count individual chromosomes belonging to different genomes by labelling the genomic DNA of the donor species with variously coloured fluorescent tags (Rayburn and Gill 1986; Zhang *et al.* 2004). These techniques also assist in detection of any chromosomal structural changes such as translocations or deletions in the recombinant hexaploid–tetraploid progeny (Schwarzacher *et al*. 1989; Lim *et al*. 2003; Eberhard *et al*. 2010; Padmanaban *et al*. 2017*b*).

In this study, we investigated the retention of D-genome chromosomes in different hexaploid–tetraploid wheat crosses over multiple generations by using both molecular and cytological approaches. Crosses having the same hexaploid maternal parent but different tetraploid paternal parents and those having different hexaploid maternal parents but the same tetraploid paternal parents were used to examine differences in D chromosome retention in the F_2 generation. Stability of the D genome was examined in one selected cross, across multiple generations up to the F_6. Any effect of the time of crossing on the retention pattern of individual D chromosomes was also examined.

Materials and methods

Plant materials

The hexaploid maternal parents used in the hexaploid–tetraploid crosses included lines B34 and B41 from a Sunco/2-49 (S2) doubled haploid (DH) population, referred to as S2B34 and S2B41, respectively, and lines B44 and E25 from a 2-49/W21MMT70 (2W) DH population, referred to as 2WB44 and 2WE25, respectively. These were crossed with tetraploid wheat lines 950329, also known as parent B, and Caparoi. The DH hexaploid lines have previously been identified as sources of partial resistance for crown rot disease caused by the fungus *Fusarium pseudograminearum* (Bovill *et al*. 2010). Crosses were conducted in the glasshouse during 2010 following the traditional method of hand emasculation and pollination (Riley and Chapman 1967). Six different hexaploid–tetraploid crosses were made to test the differences in the retention of D chromosomes in (*i*) crosses made 7 days apart (two populations S2B34/950329-1 and S2B34/950329-2); (*ii*) crosses having the same hexaploid maternal parent but different tetraploid paternal parents (S2B34/950329, S2B34/Caparoi); and (*iii*) crosses having different hexaploid maternal parents but the same tetraploid paternal parent (S2B34/Caparoi, S2B41/Caparoi; S2B34/950329, 2WB44/950329, 2WE25/950329).

Advancing F_1 and F_2 progeny and DNA extraction

The F_1 seeds were grown at room temperature in 24-well tissue culture plates containing 2% water agar. One-week-old seedlings were transferred to 200-mm-diameter plastic pots containing Searles premium grade potting mix (Advanced) (Searles, Kilcoy, Qld) and grown in the glasshouse providing optimum growing conditions. Three-week-old leaf tissues were collected and DNA was extracted by using a Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. DNA was quantified with an Implen NanoPhotometer® (Implen GmbH, Schatzbogen, Germany) and DNA quality was confirmed by gel electrophoresis using a 1% agarose gel. Microsatellite markers cfa2278 (2B) and gwm630 (2B) showed polymorphism between the parents and were used to validate the F_1 lines for heterozygosity. The F_1 plants were grown to maturity and allowed to self-pollinate for F_2 seed collection.

On average, 30 F_2 seeds were harvested from each of the six hexaploid–tetraploid crosses. The 180 F_2 lines were grown in the glasshouse during 2014. DNA was extracted from 3-week-old leaf tissue; DNA quantification and quality assessments were carried out as mentioned above. To determine the retention of D chromosomes in the F_2 generation, 180 lines derived from the six hexaploid–tetraploid crosses were assessed by utilising a set of 21 microsatellite markers covering all seven D chromosomes as described in Padmanaban *et al*. (2017*a*).

GISH analysis of F_2 lines of the 2WE25/950329 cross

Twenty F_2 lines from the cross 2WE25/950329 were assessed by use of GISH to confirm the molecular marker results with regard to the number of D chromosomes retained. Root squashes were conducted and chromosome numbers were counted via the acetocarmine staining technique as outlined in Zhang *et al*. (2004). For the GISH analysis, total genomic DNA was extracted from *T. urartu* (the A genome donor) and *Aegilops tauschii* (the D genome donor), using a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany), and labelled with fluorescein-12-dUTP and tetramethylrhodamine-5-dUTP (Roche Diagnostics, Basel, Switzerland), respectively, using nick translation. The labelling reaction was carried out at 15°C for 2 h followed by 65°C for 7 min to deactivate the enzymes. A QIAquick Nucleotide

Removal Kit (Qiagen) was used post-labelling to remove excess nucleotides. Unlabelled total genomic DNA of *Ae. speltoides* (the B-genome donor) was used as a blocker. The probe : blocker ratio was ~1 : 10. The labelled probe was hybridised with the samples, and post-hybridisation washing steps were followed as described in Zhang *et al*. (2004). Chromosomes were counterstained with 4 ,6-diamidino-2-phenylindole (Invitrogen Life Science, Carlsbad, CA, USA). Slides were analysed with an Axio Imager epifluorescence microscope (Zeiss International, Oberkochen, Germany). Images were captured with a Retiga Exi CCD (charge-coupled device) camera (QImaging, Surrey, BC, Canada) operated with Image-Pro Plus version 7.0 software (Media Cybernetics, Bethesda, MD, USA) and processed with Photoshop version 8.0 (Adobe Systems, San Jose, CA, USA).

Marker analysis of F_3 and F_4 lines of the 2WE25/950329 cross

Twenty-five F_3 seeds were selected from four individual F_2 plants of the 2WE25/950329 cross (five seeds from line 2WE25/B-3, eight from line 2WE25/B-8, six from line 2WE25/B-18, six from line 2WE25/B-26) that had retained at least one copy of each of the seven D-genome chromosomes based on the microsatellite analysis in the F_2 generation. These seeds were grown in the glasshouse under optimum growth conditions, and DNA was extracted from 1-week-old leaf tissue following the CTAB method (Saghai-Maroof *et al*. 1984). DNA samples were diluted to 50 ng μL^{-1} and sent to Diversity Arrays Technology, Canberra, ACT, for DArTseq analysis. The hexaploid wheat array with a service tag DW15-61 was used as the marker source. Individual markers were scored as 1 or 0 based on the presence or absence, respectively, of the unique DArT sequence. Quality control of DArT sequences was conducted as described in Padmanaban *et al*. (2017*a*). The F_3 lines that retained at least one copy of each of the seven D chromosomes, based on DArTseq marker results in the F_3 generation (2WE25/B-3.3, 2WE25/B-8.8, 2WE25/B-18.5, 2WE25/B-26.5), were advanced to the F_4 generation. Five seeds from each line, i.e. 20 F_4 progenies were characterised with the same set of microsatellite markers used in the analysis described above. Four F_4 lines (2WE25/B-3.3.1, 2WE25/B-8.8.1, 2WE25/B-18.5.1, 2WE25/B-26.5.1) that retained at least one copy of each of the seven D-genome chromosomes were selected and advanced to the F_5 generation for FISH analysis.

FISH analysis of F_5 generation of the 2WE25/950329 cross

For FISH analysis, probes were labelled with tetramethyl-rhodamine-5-dUTP (Roche Diagnostics) using nick translation. The D-genome chromosomes were identified with the pAs1 clone (containing D-genome-specific tandem repetitive sequences isolated from *Ae. tauschii*), whereas A-genome chromosomes were identified with the BAC clone 676D4 containing A-genome-specific dispersed repetitive sequences. Labelling and visualisation of the chromosomes was performed as indicated for GISH analysis. The D-genome chromosomes were karyotyped by using an ideogram showing unique signal patterns for each D chromosome (Supplementary materials figure 1, as available at the journal's website; Mukai *et al*. 1993; Zhang *et al*. 2004). Multiple images from the same slide were processed by Photoshop CC (Adobe Creative Cloud version 13.7.1; Adobe Systems). Ten randomly selected seeds from three F_5 lines (2WE25/B-3.3.5, 2WE25/B-18.5.3, 2WE25/B-26.5.4) were advanced to the F_6 generation to determine the retention of D-genome chromosomes by using the set of microsatellite markers described above.

Statistical analys3s

In order to identify differences in the retention of D chromosomes between different hexaploid–tetraploid crosses using data obtained from microsatellite marker analysis, multiple pair-wise comparisons were conducted by applying the Tukey–Kramer honest significant difference (HSD) test in JMP version 12 (SAS Institute, Cary, NC). To compare the proportions of chromosomes A and B inherited in four F_3 families from the 2WE25/950329 cross, a Mann–Whitney U test was performed using SPSS version 22 (IBM, Armonk, NY, USA). Graphs were drawn with Prism version 5.01 (GraphPad Software, San Diego, CA).

Results

Presence of D chromosomes in the F_2 generation of hexaploid–tetraploid crosses

In 180 F_2 progeny plants derived from six different hexaploid–tetraploid crosses, microsatellite markers could confirm the presence or absence of each unique D chromosome, but they could not be used to distinguish the presence of one or two copies. Differences in the retention of unique D chromosomes between hexaploid–tetraploid crosses in the F_2 generation are indicated in Table 1. Table 2 illustrates the total number of D chromosomes retained in each of these crosses.

Independent populations of the same cross

We observed no significant difference in the mean overall retention of D chromosomes (Table 1) between the two independent populations of the same crosses made on different days ($P = 0.341$). The percentages of mean overall retention of D-genome chromosomes in the progeny of two independent S2B34/950329 crosses were 60% and 68% (Table 1).

Effect of tetraploid paternal parent on D-genome retention

No significant difference ($P = 0.998$) in the mean overall retention of D-genome was observed in hexaploid–tetraploid crosses when different tetraploid paternal parents, 950329 and Caparoi, were used with the same hexaploid maternal parent, S2B34 (Fig. 1). The percentages of D chromosomes retained in the two S2B34/950329 crosses and the cross SB34/Caparoi were 60%, 68% and 69%, respectively (Table 1). Five and three lines (17% and 13%) in the S2B34/950329-1 and -2 crosses, respectively, and four lines (13%) in the S2B34/Caparoi cross retained at least one copy of all seven D chromosomes (Table 2). None of the lines lost all seven D chromosomes in the S2B34/950329 cross, whereas one line (3%) in the S2B34/Caparoi cross lost all D chromosomes and had a stable tetraploid number of chromosomes (2n = 28) (Table 2).

Effect of hexaploid maternal parent on D-genome retention

No significant difference ($P = 0.919$) was observed in the mean overall retention of D chromosomes between the hexaploid

Table 1. Percentage of unique D chromosomes retained in 180 F_2 lines belonging to six different hexaploid–tetraploid crosses

Unique D chromosomes	DH Sunco/2-49 (S2)				DH 2-49/W21MMT70 (2W)	
	S2B34/ 950329-1	S2B34/ 950329-2	S2B34/ Caparoi	S2B41/ Caparoi	2WB44/ 950329	2WE25/ 950329
1D	70%	61%	52%	52%	69%	87%
2D	63%	74%	74%	58%	78%	77%
3D	50%	57%	74%	58%	78%	84%
4D	63%	57%	65%	58%	66%	90%
5D	60%	78%	74%	64%	72%	87%
6D	53%	65%	71%	52%	84%	84%
7D	63%	83%	74%	48%	72%	77%
No. of lines tested	30	23	31	33	32	31
Av. % D chromosomes retained	60.28%	67.85%	69.14%	55.71%	74.14%	83.71%

Table 2. Distribution of number of copies of D chromosomes retained in 180 F_2 lines of different hexaploid–tetraploid crosses

Cross	No. of D-genome chromosomes retained:								Total no.
	Null	1	2	3	4	5	6	7	of lines
S2B34/950329	0	4	3	3	6	5	4	5	30
S2B34/950329	0	0	2	4	3	6	5	3	23
S2B34/Caparoi	1	5	2	7	2	5	5	4	31
S2B41/Caparoi	2	2	3	6	2	3	8	7	33
2WB44/950329	1	1	4	2	5	2	8	9	32
2WE25/950329	0	0	3	0	2	3	8	15	31

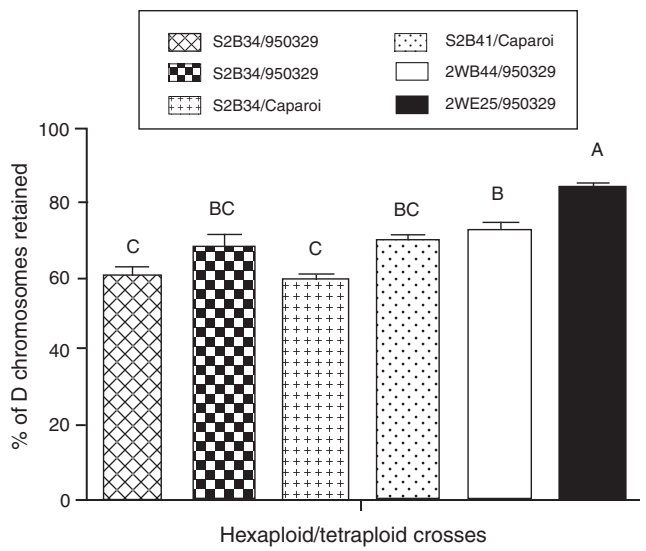


Fig. 1. Average number of D chromosomes retained (expressed as a percentage of total number of D chromosomes) in six different hexaploid–tetraploid crosses. Means (bars) with the same letter are not significantly different at $P = 0.05$. Standard errors are also presented.

parents (S2B34 and S2B41) crossed with the tetraploid line Caparoi (Fig. 1). However, a significant difference ($P = 0.0199$) in mean overall retention of D chromosomes was identified between hexaploid maternal parents (2WB44, 2WE25) crossed with the tetraploid paternal line 950329 (Fig. 1).

Among the different hexaploid–tetraploid crosses, the cross 2WE25/950329 was identified as exceptional in terms of overall mean retention of D-genome chromosomes (Fig. 1) compared with the other crosses, with 84% of D chromosomes retained and 15 of 31 lines (48%) having at least one copy of each of the seven D chromosomes (Table 2).

GISH analysis of the F_2 generation of the 2WE25/950329 cross

To confirm the ability of the 2WE25/950329 cross to retain a significant number of D-genome chromosomes, we used GISH to assess 20 more F_2 lines from the same cross. The chromosome number estimated for the 2WE25/950329 cross ranged from 30 to 40, with 70% of the lines (14 of 20) having $\geq$35 chromosomes (Table 3). The four lines with 40 chromosomes either contained two copies of each of six unique D chromosomes or possessed a single copy of two unique D chromosomes in addition to two copies of the other five D chromosomes. Telocentric D chromosomes were observed in two of 20 lines (Fig. 2). The GISH results confirmed the potential of the 2WE25/950329 cross to retain a large number of D chromosomes. Thus, we selected F_2 lines 2WE25/B-3, 2WE25/B-8, 2WE25/B-18 and 2WE25/B-26 from this cross for further molecular and cytological assessment to investigate the retention of D chromosomes in the subsequent F_3 generation. These lines each contained at least one copy of each D chromosome.

DArT analysis of the F_3 progenies of the 2WE25/950329 cross

The retention of D chromosomes and inheritance of A and B chromosomes from the hexaploid wheat parent 2WE25 were determined in 25 F_3 progeny from four individual F_2 plants of the 2WE25/B cross that had retained at least one copy of each of the seven D-genome chromosomes based on the microsatellite

Table 3. Results of genomic *in situ* hybridisation (GISH) analysis indicating chromosome numbers in 20 F_2 lines of the 2WE25/950329 cross

No. of chromosomes	No. of F_2 lines
28	0
29	0
30	2
31	0
32	2
33	2
34	0
35	2
36	2
37	1
38	2
39	3
40	4
41	0
42	0
Total no. of F_2 lines	20

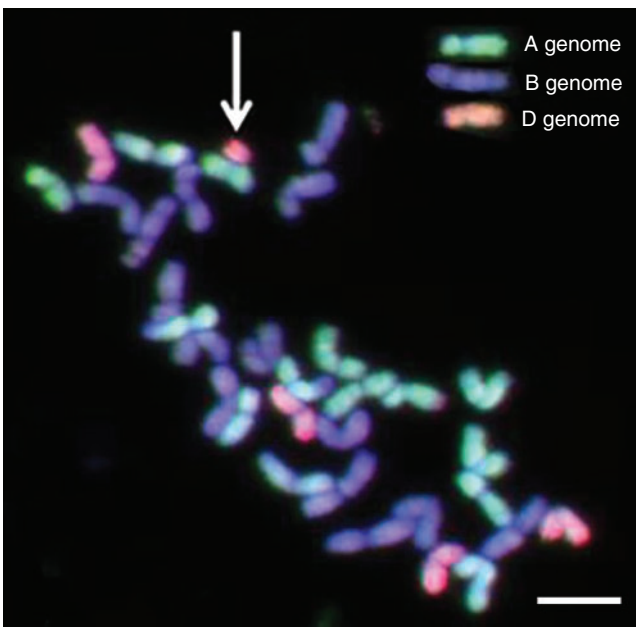


Fig. 2. Multi-colour genomic *in situ* hybridisation (GISH) analyses of two F_2 lines of the 2WE25/950329 cross. Genomic DNA of *Triticum urartu* (A-genome donor) and *Aegilops tauschii* (D-genome donor) were labelled with different fluorescence tags, FITC (green) and tetramethylrhodamine (pink), respectively. Unlabelled genomic DNA from *Ae. speltoides* (B-genome donor) was used as a blocker and counter-stained with DAPI (blue). Arrow indicates the presence of a telocentric D chromosome in an F_2 line of the 2WE25/950329 cross. Scale bar is equal to 10 µm.

analysis in the F_2 generation (Table 4). Genome-wide, high-density DArTseq markers were used to characterise the four distinct families of the 2WE25/950329 cross (2WE25/B-3, 2WE25/B-8, 2WE25/B-18, 2WE25/B-26). Of 3006 unique DNA sequences, 1556 sequences were generated across the seven D chromosomes, and 614 and 836 sequences were generated on the A and B chromosomes, respectively. Of the 1450 polymorphic DArTseq markers on chromosomes A and B, 702 (48%) indicated a positive signal on the tetraploid parent.

The DArTseq analysis indicated that 44% of lines had retained at least one copy of all seven D chromosomes in the F_3 generation (Table 4). Furthermore, 36% of the progenies tested retained at least one copy of six unique D chromosomes, 16% retained at least one copy of five unique D chromosomes, and the remaining 4% retained at least four unique D chromosomes. Chromosome 4D was present in every line assessed, whereas chromosome 6D was retained at a lower rate, with 19 of 25 lines (76%) having this chromosome. We also observed the complete absence of the 1D long arm, 2D short arm and 6D long arm in lines E25/B-3.1, E25/B-8.7 and E25/B-18.2, respectively.

Mean average overall proportions of A and B chromosomal sequences were inherited in a 1 : 1 ratio for hexaploid : tetraploid wheat in the pentaploid-derived F_3 progenies of the 2WE25/950329 cross. However, individual lines showed differences in the mean average proportion of hexaploid wheat alleles inherited, ranging from 37% to 62% and from 40% to 59.9% in chromosomes A and B, respectively (Table 4). A significant difference in the proportion of A- and B-genome alleles inherited from bread wheat was observed between most of the F_3 families (Table 5). Five randomly selected seeds of each of the four selected F_3 lines were investigated for D-genome retention in the F_4 generation by using microsatellite markers. Lines that retained at least one copy of each of the seven D chromosomes were advanced to the F_5 generation.

FISH assessment of the F_5 generation of the 2WE25/950329 cross

A FISH analysis was carried out on the F_5 generation to estimate the number of unique paired D chromosomes that were retained. Karyotyping of the 20 F_5 lines was carried out based on the distinct FISH signals generated from pAs1 on each D-genome chromosome. The example in Fig. 3*a* shows line 2WE25/B-8.8.4, which had only one copy of chromosome 6D. FISH results indicated that 13 of 20 (65%) lines had retained at least one copy of each of the seven unique D-genome chromosomes (Table 6). All 20 lines had retained two copies of chromosome 4D, followed by chromosome 5D with at least one copy. FISH results further revealed that chromosome 5D of line 2WE25/B-3.3.5 had divided at the centromere region and that the short and long arms were inherited separately as telocentric chromosomes (Fig. 3*b*).

Among the four different lines evaluated at the F_5 generation, 99% of the progeny of line 2WE25/B-8 retained both copies of all seven D chromosomes (Table 6). In the remaining three lines, D chromosomes had randomly been eliminated with a loss of 13% (2WE25/B-3.3), 9% (2WE25/B-18.5) and 23% (2WE25/B-26.5). Overall, 15% of D chromosomes were eliminated between the F_4 and F_5 generations (Tables 4 and 6). Ten seeds were randomly selected from each of three F_5 lines (2WE25/B3.3.5, 2WE25/B-18.5.3, and 2WE25/B-26.5.4) and were advanced to F_6 generation. This included line 2WE25/B-3.3.5 with telocentric chromosome 5D.

Table 4. Retention of D chromosomes in 31 F_2 and 25 F_3 progeny of the 2WE25/950329 cross

Durum line 950329 is referred to as 'B'. Each column represents an individual line and each row a unique chromosome. Red and yellow cells show the presence and absence of D chromosomes, respectively. F_2 lines advanced to the F_3 generation are indicated by different shades of grey. DArT analysis of mean overall percentage of A and B bread wheat alleles inherited in the F_3 generation is indicated by red, orange and yellow cells containing proportions of 70–100%, 40–70% and 0–40%, respectively

F_2 Lines	2WE25/B-1	2WE25/B-2	2WE25/B-3	2WE25/B-4	2WE25/B-5	2WE25/B-6	2WE25/B-7	2WE25/B-8	2WE25/B-9	2WE25/B-10	2WE25/B-11	2WE25/B-12	2WE25/B-13	2WE25/B-14	2WE25/B-15	2WE25/B-16	2WE25/B-17	2WE25/B-18	2WE25/B-19	2WE25/B-20	2WE25/B-21	2WE25/B-22	2WE25/B-23	2WE25/B-24	2WE25/B-25	2WE25/B-26	2WE25/B-27	2WE25/B-28	2WE25/B-29	2WE25/B-30	2WE25/B-31
1D																															
2D																															
3D																															
4D																															
5D																															
6D																															
7D																															

F_3 Lines	2WE25/B-3.1	2WE25/B-3.2	2WE25/B-3.3	2WE25/B-3.4	2WE25/B-3.5	2WE25/B-8.1	2WE25/B-8.2	2WE25/B-8.3	2WE25/B-8.4	2WE25/B-8.5	2WE25/B-8.6	2WE25/B-8.7	2WE25/B-8.8	2WE25/B-18.1	2WE25/B-18.2	2WE25/B-18.3	2WE25/B-18.4	2WE25/B-18.5	2WE25/B-18.6	2WE25/B-26.1	2WE25/B-26.2	2WE25/B-26.3	2WE25/B-26.4	2WE25/B-26.5	2WE25/B-26.6
1D																									
2D																									
3D																									
4D																									
5D																									
6D																									
7D																									
1A	65	43	67	58	63	69	73	59	70	59	69	65	70	33	30	25	29	29	22	51	74	62	62	62	65
2A	76	75	76	75	69	31	35	35	33	41	33	33	41	39	61	68	35	74	57	33	33	40	33	34	36
3A	15	36	32	70	21	72	65	57	60	69	65	62	60	38	34	37	35	36	41	51	73	48	65	77	73
4A	54	75	61	63	52	42	42	50	45	47	43	43	45	71	56	59	33	54	40	43	37	48	40	46	40
5A	32	42	37	38	30	67	54	63	58	61	63	58	79	74	70	57	67	74	54	60	80	64	59	76	73
6A	33	27	31	39	27	25	65	35	35	77	81	61	35	33	30	27	31	28	28	65	70	46	47	57	72
7A	23	22	33	17	38	73	48	62	54	75	57	47	62	50	37	44	32	52	40	69	67	45	68	46	70
1B	25	37	35	37	32	70	69	78	82	66	63	70	76	33	34	36	37	33	35	57	84	63	70	65	71
2B	80	80	88	82	81	50	58	50	52	50	42	50	50	59	44	36	35	67	43	45	47	41	47	49	49
3B	39	54	54	47	46	73	68	75	75	73	75	73	71	63	67	61	64	62	64	62	67	68	65	73	72
4B	19	20	34	39	42	43	40	47	41	52	39	47	42	36	25	42	36	47	44	58	41	57	52	42	44
5B	25	48	56	46	25	66	28	57	66	49	53	55	31	41	42	46	45	47	41	47	43	30	49	48	22
6B	41	41	30	44	36	63	70	48	49	75	85	81	57	26	37	31	32	27	45	59	61	69	58	58	64
7B	90	84	80	83	81	47	52	48	43	46	45	40	38	34	27	30	40	42	32	39	37	35	48	49	35

Retention of D-genome chromosomes in F_6 generation of the 2WE25/950329 cross

Thirty F_6 progenies derived from three selected F_5 lines were screened with microsatellite markers to determine the presence or absence of D chromosomes. Line 2WE25/B-3.3.5 had a complete set of telocentric chromosome 5D and only one copy of each of chromosomes 6D and 7D in the F_5 generation. In the F_6 generation, this line's progeny had only the long arm of chromosome 6D in all 10 lines (Table 7). Five of the 10 lines retained a copy of chromosome 7D, whereas one line had lost both copies of 7D and the remaining four lines only had the long arm of chromosome 7D. Three of the 10 F_6 lines had lost the 5D chromosomes, whereas the remaining seven lines had inherited both arms of this chromosome in the F_6 generation (Table 7).

Table 5. Significant differences between F_3 families of the 2WE25/950329 cross with regard to the proportion of A and B chromosome bread wheat alleles inherited

*$P < 0.05$; **$P < 0.01$; n.s., not significant ($P > 0.05$)

	2WE25/B-3	2WE25/B-8	2WE25/B-18
	A genome		
2WE25/B-8	0.008**	–	
2WE25/B-18	0.476n.s.	0.01*	–
2WE25/B-26	0.019*	0.755n.s.	0.002**
	B genome		
2WE25/B-8	0.006**	–	
2WE25/B-18	0.009**	0.001**	–
2WE25/B-26	0.247n.s.	0.020*	0.002**

The F_5 line 2WE25/B-18.5.3 retained two copies of all seven D chromosomes in the F_6 generation. This was expected because this line had a stable hexaploid wheat chromosomal number of 2n = 42.

Line 2WE25/B-26.5.4 did not have the chromosome 2D and only one copy of each of chromosomes 3D and 6D in the F_5 generation. In the F_6 generation, two of the 10 lines showed a complete absence of the 6D chromosomes, whereas another three lines inherited only the long arm of chromosome 6D.

Discussion

This is the first study, to our knowledge, to employ both cytological and molecular techniques in studying the presence or absence of D-genome chromosomes through multiple generations of a hexaploid–tetraploid cross. No significant differences were observed in independent crosses having the same parents and crosses having the same maternal parent crossed with two different paternal parents (950329 and Caparoi). However, we observed a significant difference in the retention of D-genome chromosomes between crosses involving different hexaploid wheat parents, 2WB44, 2WE25 and S2B34, crossed with tetraploid wheat parent 950329. These observations suggest that the hexaploid maternal parent played a role in determining the retention of D chromosomes in the F_2 generation in these sets of hexaploid–tetraploid crosses. Similar findings were presented by Martin *et al*. (2011), where differences in the retention of D-genome chromosomes were observed between hexaploid–tetraploid crosses.

The mean overall retention of D chromosomes was calculated as 84% in the F_2 generation of the 2WE25/950329 cross, which

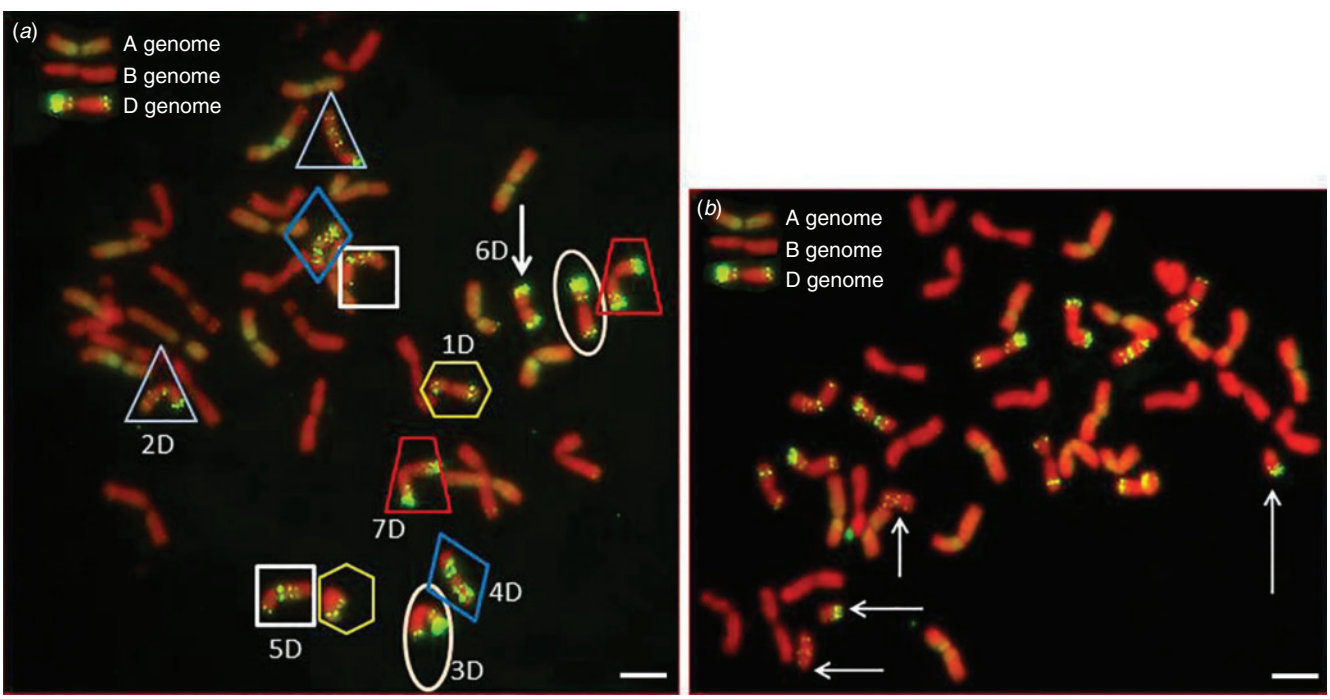


Fig. 3. Fluorescence *in situ* hybridisation (FISH) analyses of mitotic metaphase chromosomes of a F_5 line of the 2WE25/950329 cross: (*a*) karyotype of line 2WE25/B-8.8.4, with D chromosome pairs indicated by enclosure in the same shape; (*b*) telocentric 5D chromosomes observed in line 2WE25/B-3.3.5. Scale bar is equal to 10 µm.

Table 6. Fluorescence *in situ* hybridisation (FISH) analysis indicating the number of unique D chromosomes retained in the F_5 generation of the 2WE25/950329 cross

Durum line 950329 is referred to as 'B'. Delineated rows represent unique D-genome chromosomes in homologous pairs; each column represents an individual F_5 line. Red and yellow cells show the presence and absence of D chromosomes, respectively

	2WE25/B-3.3.1 to B-3.3.5					2WE25/B-8.8.1 to B-8.8.5					2WE25/B-18.5.1 to B-18.5.5					2WE25/B-26.5.1 to B-26.5.5				
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
1D																				
2D																				
3D																				
4D																				
5D																				
6D																				
7D																				

Table 7. Retention of D chromosomes in 30 selected F_6 lines of the 2WE25/950329 cross

Durum line 950329 is referred to as 'B'. Each row represents a unique D chromosome and each column an individual F_6 line. Red and yellow cells show the presence and absence of D chromosomes, respectively. S, Short arm; C, centromere; L, long arm

		2WE25/B-3.3.5.1 to B-3.3.5.10										2WE25/B-18.5.3.1 to B-18.5.3.10										2WE25/B-26.5.4.1 to B-26.5.4.10									
		1	2	3	4	5	6	7	8	9	10	1	2	3	4	5	6	7	8	9	10	1	2	3	4	5	6	7	8	9	10
1D	S																														
	C																														
	L																														
2D	S																														
	C																														
	L																														
3D	S																														
	C																														
	L																														
4D	S																														
	C																														
	L																														
5D	S																														
	C																														
	L																														
6D	S																														
	C																														
	L																														
7D	S																														
	C																														
	L																														

was significantly higher than in the other crosses tested in this study. We also observed that derivatives of this cross retained at least one copy of a complete set of seven D chromosomes in 48% of lines (15 of 31), also a significantly higher percentage than the rest of the crosses investigated. GISH analysis of this cross confirmed the presence of a high number of chromosomes (>35) in 14 of 20 lines (60%) assessed.

Marker analysis indicated that a significant number of D chromosomes were still present in the F_3 and F_4 generations of the 2WE25/950329 cross even though we had deliberately selected for all D chromosomes. Because markers cannot be used to distinguish the presence of one or two copies of a D chromosome, the monosomic or disomic status of the D chromosome set of a line cannot be assumed without further cytological analysis of subsequent generations. Results from the FISH analyses in the F_5 generation of the 2WE25/950329 cross suggest that none of the lines having at least one copy of all seven D chromosomes in the F_3 generation had a complete paired set of 14 D chromosomes, because univalent D chromosomes were identified in most lines of the F_5 generation. These results further suggest that these lines from this cross remain unstable at the F_5 generation, which is contrary to the report by Kihara (1924), who suggested that most pentaploid-derived hexaploid–tetraploid wheat hybrids stabilise to a complete hexaploid (2n = 42) or tetraploid (2n = 28) D-genome set by the F_5 generation. It is unknown whether paired D chromosomes that do not form a complete D-genome set and are still present at the F_5 and F_6 generations become stable in subsequent generations. This needs to be studied by cytological testing of non-segregating lines. Interestingly in the present study, chromosome 4D was inherited as a homologous pair in all lines assessed in the F_5 and F_6 generations. These results are similar to a previous report in which the majority (75%) of randomly selected F_2 lines of an LRC2010-150/WID802 cross had retained chromosome 4D (Padmanaban *et al*. 2017*a*). Preferential retention of chromosome 4D in hexaploid–tetraploid crosses needs further confirmation by assessing several other hexaploid–tetraploid crosses.

Unpaired D chromosomes seem to undergo several different paths during meiosis, with some being eliminated, some being doubled to form a bivalent set, and some undergoing a centric break resulting in telocentric chromosomes. One line of the 2WE25/B-3.3.5 cross had both telocentric long and short arms of chromosome 5D in the F_5 generation. These telocentric chromosomes were again observed in the F_6 generation in seven of 10 progeny. Telocentric D chromosomes were also identified in two of 20 lines in the F_2 generation of the 2WE25/950329 cross. In these instances, the seedset was poor and these lines could not be followed for further investigations. Inheritance of telocentric chromosomes with a terminal centromere has rarely been observed in plants. It has been proposed that the stability and inheritance of telocentric chromosomes is predominantly based on centromere size and the degree of completeness of its kinetochore (Koo *et al*. 2015).

Researchers developing elite bread wheat lines from pentaploid crosses need to be proactive in selecting lines that retain at least one copy of each D chromosome in the F_2 generation. Although the 2WE25/B cross retained a substantial proportion of D-genome chromosomes in the F_2 generation, it eventually showed elimination of the unpaired D chromosomes in the F_5 generation. Backcrossing these lines with the bread wheat parent would have increased the probability of hexaploid genome stability in a subset of lines of the 2WE25/B cross in the subsequent generations. By contrast, the tendency toward rapid D chromosome elimination in many lines derived from the hexaploid–tetraploid wheat crosses in the present study would be of use to durum breeders, by providing the opportunity for incorporation of selected traits of the A and B genomes of bread wheat into a stable tetraploid genome after only a few generations. Overall, this study demonstrates the feasibility of selecting novel genetic recombinations in hexaploid–tetraploid crosses that will aid plant breeders in further improving elite bread and durum wheat lines.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgement

This study was partly funded by the Grains Research and Development Corporation, Australia. Sriram Padmanaban acknowledges the support of an Australian Postgraduate Award.

References

Bovill WD, Horne M, Herde D, Davis M, Wildermuth GB, Sutherland M (2010) Pyramiding QTL increases seedling resistance to crown rot (*Fusarium pseudograminearum*) of wheat (*Triticum aestivum*). *Theoretical and Applied Genetics* **121**, 127–136. doi:10.1007/s00122-010-1296-7

Eberhard FS, Zhang P, Lehmensiek A, Hare RA, Simpfendorfer S, Sutherland MW (2010) Chromosome composition of an F_2 *Triticum aestivum* × *T. turgidum* spp. *durum* cross analysed by DArT markers and MCFISH. *Crop & Pasture Science* **61**, 619–624. doi:10.1071/CP10131

Gilbert J, Procunier J, Aung T (2000) Influence of the D genome in conferring resistance to fusarium head blight in spring wheat. *Euphytica* **114**, 181–186. doi:10.1023/A:1004065620127

Kalous J, Martin J, Sherman J, Heo H-Y, Blake N, Lanning S, Eckhoff J, Chao S, Akhunov E, Talbert L (2015) Impact of the D genome and quantitative trait loci on quantitative traits in a spring durum by spring bread wheat cross. *Theoretical and Applied Genetics* **128**, 1799–1811. doi:10.1007/s00122-015-2548-3

Kihara H (1924) 'Cytologische und genetische Studien bei wichtigen Getreidearten mit besondere Riichsicht auf das Verhalten der Chromosomen und die Sterilitat in den Bastarden.' Memoirs of the College of Science, University of Kyoto. (Faculty of Science, University of Kyoto: Kyoto, Japan)

Kihara H (1982) 'Wheat studies: retrospect and prospects.' (Kodansha Ltd: Tokyo)

King J, Armstead I, Harper J, Ramsey L, Snape J, Waugh R, James C, Thomas A, Gasior D, Kelly R (2013) Exploitation of interspecific diversity for monocot crop improvement. *Heredity* **110**, 475–483. doi:10.1038/hdy.2012.116

Koo D-H, Sehgal SK, Friebe B, Gill BS (2015) Structure and stability of telocentric chromosomes in wheat. *PLoS One* **10**, e0137747. doi:10.1371/journal.pone.0137747

Lanning SP, Blake NK, Sherman JD, Talbert LE (2008) Variable production of tetraploid and hexaploid progeny lines from spring wheat by durum wheat crosses. *Crop Science* **48**, 199–202. doi:10.2135/cropsci2007.06.0334

Lim K-B, Ramanna MS, Jacobsen E, van Tuyl JM (2003) Evaluation of BC2 progenies derived from 3x-2x and 3x-4x crosses of *Lilium* hybrids: a GISH

analysis. *Theoretical and Applied Genetics* **106**, 568–574. doi:10.1007/s00122-002-1070-6

Martin A, Simpfendorfer S, Hare RA, Eberhard FS, Sutherland MW (2011) Retention of D genome chromosomes in pentaploid wheat crosses. *Heredity* **107**, 315–319. doi:10.1038/hdy.2011.17

Martin A, Simpfendorfer S, Hare R, Sutherland M (2013) Introgression of hexaploid sources of crown rot resistance into durum wheat. *Euphytica* **192**, 463–470. doi:10.1007/s10681-013-0890-6

Mukai Y, Nakahara Y, Yamamoto M (1993) Simultaneous discrimination of the three genomes in hexaploid wheat by multicolor fluorescence *in situ* hybridization using total genomic and highly repeated DNA probes. *Genome* **36**, 489–494. doi:10.1139/g93-067

Munns R, Hare R, James R, Rebetzke G (2000) Genetic variation for improving the 648 salt tolerance of durum wheat. *Australian Journal of Agricultural Research* **51**, 69–74. doi:10.1071/AR99057

Padmanaban S, Sutherland MW, Knight NL, Martin A (2017*a*) Genome inheritance in populations derived from hexaploid/tetraploid and tetraploid/hexaploid wheat crosses. *Molecular Breeding* **37**, 48. doi:10.1007/s11032-017-0647-3

Padmanaban S, Zhang P, Hare RA, Sutherland MW, Martin A (2017*b*) Pentaploid wheat hybrids: Applications, characterisation, and challenges. *Frontiers in Plant Science* **8**, 358. doi:10.3389/fpls.2017.00358

Rayburn AL, Gill BS (1986) Isolation of a D-genome specific repeated DNA sequence from Aegilops squarrosa. *Plant Molecular Biology Reporter* **4**, 102–109. doi:10.1007/BF02732107

Ren R, Ray R, Li P, Xu J, Zhang M, Liu G, Yao X, Kilian A, Yang X (2015) Construction of a high-density DArTseq SNP-based genetic map and identification of genomic regions with segregation distortion in a genetic population derived from a cross between feral and cultivated-type watermelon. *Molecular Genetics and Genomics* **290**, 1457–1470. doi:10.1007/s00438-015-0997-7

Riley R, Chapman V (1967) The inheritance in wheat of crossability with rye. *Genetical Research* **9**, 259–267. doi:10.1017/S0016672300010569

Saghai-Maroof MA, Soliman KM, Jorgensen RA, Allard R (1984) Ribosomal DNA spacer-length polymorphisms in barley: Mendelian inheritance, chromosomal location, and population dynamics. *Proceedings of the National Academy of Sciences of the United States of America* **81**, 8014–8018. doi:10.1073/pnas.81.24.8014

Schwarzacher T, Leitch A, Bennett M, Heslop-Harrison J (1989) *In situ* localization of parental genomes in a wide hybrid. *Annals of Botany* **64**, 315–324. doi:10.1093/oxfordjournals.aob.a087847

Sharma HC, Gill BS (1983) Current status of wide hybridization in wheat. *Euphytica* **32**, 17–31.

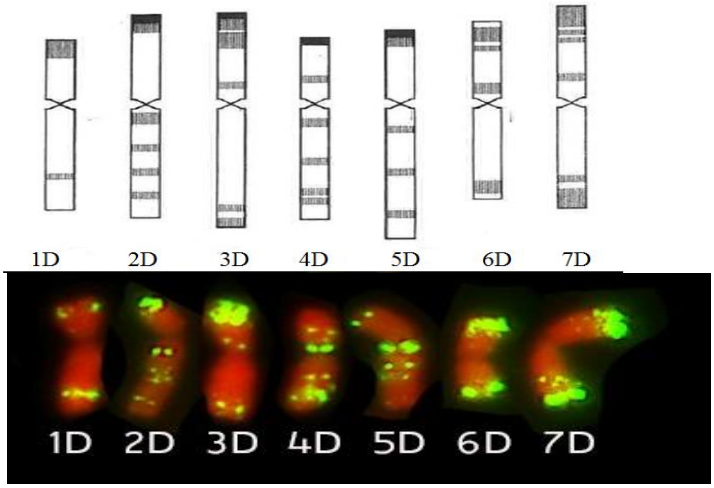
Sheedy JG, McKay AC, Lewis J, Vanstone VA, Fletcher S, Kelly A, Thompson JP (2015) Cereal cultivars can be ranked consistently for resistance to root-lesion nematodes (*Pratylenchus thornei* & *P. neglectus*) using diverse procedures. *Australasian Plant Pathology* **44**, 175–182. doi:10.1007/s13313-014-0333-4

Wang H, Liu D, Yan Z, Wei Y, Zheng Y (2005) Cytological characteristics of F_2 hybrids between *Triticum aestivum* L. and *T. durum* Desf with reference to wheat breeding. *Journal of Applied Genetics* **46**, 365–369.

Xu L, Wang M, Cheng P, Kang Z, Hulbert S, Chen X (2013) Molecular mapping of *Yr53*, a new gene for stripe rust resistance in durum wheat accession PI 480148 and its transfer to common wheat. *Theoretical and Applied Genetics* **126**, 523–533. doi:10.1007/s00122-012-1998-0

Zhang P, Li W, Friebe B, Gill BS (2004) Simultaneous painting of three genomes in hexaploid wheat by BAC-FISH. *Genome* **47**, 979–987. doi:10.1139/g04-042

Supplementary Material :



Supplementary Figure 1 Top: Ideogram of D-genome chromosomes (Grey signals, Mukai et al. 1993). Bottom: FISH analysis of D-genome chromosomes showing specific fluorescence signals from pAs1 clone hybridised on each chromosome.

Supplementary Table 1 Number of unique DArT™ sequence markers for each A, B and D chromosome, and the number of markers which hybridised to the durum parent.

Chromosomes	A	B	D
1	89 (44)	119 (67)	201
2	113 (42)	182 (84)	331
3	87 (43)	207 (94)	154
4	122 (61)	70 (43)	255
5	50 (28)	90 (40)	236
6	78 (36)	92 (43)	174
7	75 (47)	76 (30)	205
Total	614 (301)	836 (401)	1556
Average	88.71 (43)	119.42 (57)	222.28

Chapter 4

Genome inheritance in populations derived from hexaploid / tetraploid and tetraploid / hexaploid wheat crosses

The second study investigated the genome inheritance by comparing the two populations derived from hexaploid/tetraploid and tetraploid/hexaploid wheat crosses using the same parental lines. Pairs of inter-ploidy crosses between LRC2010-150 /WID802 and WID802/LRC2010-150 were developed for this study.

The objectives of study 2 were to examine the degree of inheritance of the durum A and B genome and to evaluate the differences in the retention of D-genome chromosomes between the hexaploid/tetraploid and tetraploid/hexaploid cross. This study also examined the relationship between inheritance of the durum A and B genome to the retention of D genome of hexaploid/tetraploid and tetraploid/hexaploid crosses. The content of this chapter is published in the journal “Molecular Breeding" and details are below.

Padmanaban S, Sutherland MW, Knight NL, and Martin A (2017) “Genome inheritance in populations derived from hexaploid/tetraploid and tetraploid/hexaploid wheat crosses”. **Molecular Breeding** 37:48.doi: 10.1007/s11032-017-0647-3

Note: Supplementary data associated with this chapter are attached along with the article

Mol Breeding (2017) 37:48
DOI 10.1007/s11032-017-0647-3

Genome inheritance in populations derived from hexaploid/tetraploid and tetraploid/hexaploid wheat crosses

Sriram Padmanaban · Mark W. Sutherland · Noel L. Knight · Anke Martin

Received: 25 August 2016 / Accepted: 27 February 2017

Abstract Hexaploid/tetraploid and tetraploid/hexaploid wheat hybrids were established using the hexaploid (*Triticum aestivum* L.) bread wheat LRC2010-150 and the tetraploid durum wheat (*T. turgidum* spp. *durum*) WID802. Thirty F_2 progeny from each cross were characterised using Diversity Arrays Technology (DArTseq™) markers to determine whether there are differences between the crosses in the proportion of A, B and D genomic material inherited from each parent. Inheritance of the A and B genome from the tetraploid durum parent varied from 32 to 63% among the 60 lines assessed, and results indicated significant differences between the two F_2 populations in the mean overall proportion of chromosomes A and B inherited from each parent. Significant differences were also observed between the crosses in the proportion of chromosomal segments on 2B, 3A, 3B and 4A inherited from the tetraploid parent. The F_2 populations also showed significant differences in the average retention of D chromosomes per line with the tetraploid/hexaploid cross retaining a mean of 2.83 chromosomes while the reciprocal cross retained a mean of 1.8 chromosomes per line. A strong negative correlation was observed in individual lines from both populations between the proportion of the A and B genome inherited from the tetraploid durum parent and the retention of the D genome. The implication of these results for the design of efficient crossing strategies between hexaploid and tetraploid wheats is discussed.

Keywords DArTseq™ · Hexaploid/tetraploid (or) tetraploid/hexaploid · Interploidy crosses · Pentaploid hybrids · Retention of D chromosomes

Introduction

Interspecific crossing between hexaploid bread wheat (*Triticum aestivum* L., $2n = 6x = 42$) and tetraploid durum wheat (*T. turgidum* spp. *durum*, $2n = 4x = 28$) has been recognised as a potential breeding tool to improve the genetic background of bread and durum wheat species. Bread and durum wheats have the A and B genome in common, in addition to which bread wheat possesses the D genome. The interspecific hybridisation between these two different ploidy level species results in F_1 pentaploid wheat hybrids (Kihara 1925; Padmanaban et al. 2017). Kihara (1925) characterised F_1 pentaploid wheat hybrids cytologically and established the chromosome number as being $2n = 5x = 35$, consisting of 14 bivalent (A, B) and 7 univalent (D) chromosomes. In the F_2 generation, the chromosome numbers in individual progeny varied from $2n = 28$ to $2n = 42$. He observed that in subsequent generations produced from F_2 lines without at least one copy of each D chromosome, D chromosomes were progressively eliminated in viable progeny, resulting

Electronic supplementary material The online version of this article (doi:10.1007/s11032-017-0647-3) contains supplementary material, which is available to authorized users.

S. Padmanaban · M. W. Sutherland · N. L. Knight · A. Martin (✉)
Centre for Crop Health, University of Southern Queensland, West St, Toowoomba, QLD 4350, Australia
e-mail: anke.martin@usq.edu.au

Published online: 22 March 2017

eventually in a return to the tetraploid chromosome number. In contrast, some stable hexaploid lines could be selected from subsequent generations derived from those F_2 lines that carried at least one copy of each D chromosome. The pioneering work of Kihara (1925) and subsequent groups aiming to generate successful interspecific hybrids struggled to produce fertile progeny. These difficulties included incompatibility between parental genotypes, low viability of F_1 seeds, poor seedling vigour and significant levels of sterility in F_1 plants (Sharma and Gill 1983). However, alleles for a number of desirable characters have successfully been transferred between tetraploid and hexaploid wheats (Sharma and Gill 1983). Recently, for example, improved levels of crown rot disease resistance have been introduced into durum wheats from several hexaploid sources (Martin et al. 2013). Similarly, the stripe rust resistance gene *Yr53*, present in durum wheat accession PI48018, has been transferred to hexaploid bread wheat via a cross to the cultivar Avocet (Xu et al. 2013).

Understanding the different ploidy levels of wheat species and crossing them in the right direction is critical for developing successful interspecific wheat hybrids. Studies in the past have focussed on the interspecific hybridisation of different ploidy levels in only one direction, based on hexaploid/tetraploid crosses in which the bread wheat is the female parent (Gilbert et al. 2000; Wang et al. 2005; Lanning et al. 2008; Eberhard et al. 2010). Generally, it is considered that when crossing two different ploidy level wheat species, the higher ploidy level species should be employed as the maternal parent to improve the success rate in relation to seed set and germination (Kihara 1982). Complete failure of seed set was recorded in the F_1 generation when a diploid *T. monococcum* (AA) was used as the female parent and pollinated with a tetraploid (AABB) *T. turgidum* ssp. *durum* (Bhagyalakshmi et al. 2008). Among the few studies that have focussed on tetraploid/hexploid crosses, Wang et al. (2005) successfully developed an interspecific wheat cross involving the tetraploid durum wheat line As295 as the female parent and the hexaploid bread wheat cultivar Chuannong 16 (CN16) as the male parent. The chromosome distribution was examined in 55 recombinant F_2 lines and the average chromosome number per plant was 36.54. None of the progeny had lost all seven D genome chromosomes.

Molecular markers, such as simple sequence repeat (SSR) markers, can be used to identify individual chromosomes (Gupta and Varshney 2000; Somers et al. 2004). Lanning et al. (2008) used SSR markers to distinguish the D chromosomes and quantify the variations among the recombinant lines of hexaploid/tetraploid crosses. More recently, Diversity Arrays Technology (DArT) markers, which provide a higher density of polymorphic markers that can be rapidly screened across interspecific and intraspecific plant hybrids (Kopecký et al. 2009; Ren et al. 2015), have been used to evaluate interploidy of hexaploid/tetraploid crosses (Martin et al. 2011). However, DArT analyses of the D chromosomes effectively report dominant markers, and it is not possible to determine whether one or two copies of a particular chromosome segment are present. Using a combination of marker analysis and the cytological technique multicolour fluorescence in situ hybridisation (MCFISH), Eberhard et al. (2010) were able to distinguish the true number of A, B and D chromosomes present in the progeny of interspecific crosses, determine which D chromosomes had been retained and identify rare chromosomal translocations and fragments.

Studies of the chromosome composition of pentaploid-derived lines have mainly focussed on hexaploid/tetraploid crosses (Lanning et al. 2008; Eberhard et al. 2010; Martin et al. 2011; Kalous et al. 2015). For reciprocal tetraploid/hexaploid crosses, little is known regarding the proportions of hexaploid- and tetraploid-derived material inherited in the A and B genomes, or the comparative retention of the D genome in the F_2 and subsequent F_3 generations. In this study, we employed DArTseq™ markers to compare the two populations derived from hexaploid/tetraploid and tetraploid/hexaploid crosses using the same parent lines. We report on (i) the degree of inheritance of the durum (A and B) genomes in these crosses, (ii) the retention of D chromosomes in the F_2 and F_3 generations and (iii) the relationship between the degree of inheritance of the durum genome and the retention of D genome chromosomes.

Materials and methods

Plant materials and crosses

A spring hexaploid bread wheat line, LRC2010-150 (GW95-703*C15/Lang) was crossed with a current spring tetraploid durum wheat variety, WID802 (Syrica-1/Yallaroi//Tamaroi/Lingzhi/Yallaroi*2///RAC875/Kalka//Tamaroi////Lingzhi/Yallaroi//Tamaroi///Lingzhi/Yallaroi). We are currently involved

in a program which aims to introgress, partial resistance against crown rot disease, caused by fungus *Fusarium pseudograminearum*, from bread wheat into durum. To help with this process, we need to have a better understanding of the differences in genome inheritance between hexaploid/tetraploid and tetraploid/hexaploid crosses. Thus, the bread wheat parent LRC2010-150 used in this study was selected for its partial resistance to crown rot disease. The bread and durum wheat parents were crossed in both directions, i.e. hexaploid/tetraploid and tetraploid/hexaploid, following traditional hand emasculation and pollination methods (Riley and Chapman 1967). The F_1 seeds from the pollinated heads were collected and germinated in 24-well tissue culture plates containing 2% water agar. Week-old seedlings were removed and transferred into 200-mm-diameter plastic pots containing a commercial potting mixture (Searle's® certified premium grade AS 3743 JC& AT Searle Pty Ltd., Brisbane). The plants were grown to maturity in a glass house and allowed to self-pollinate.

Genomic DNA extraction of F_1 progeny and confirmation of heterozygosity

DNA was extracted from three-week-old leaves using a Wizard Genomic DNA Purification Kit (Promega Corporation, Sydney, Australia) as per the manufacturer's instructions. The DNA of all individual F_1 progeny was checked for heterozygosity with randomly selected SSR markers wmc120 (1A), gwm408 (5B) and cfa2278 (2B), which were polymorphic in the hexaploid and tetraploid parents. To confirm the presence of all seven unique D chromosomes, three SSR markers, one on each arm and one near the centromere region, were used for each of the seven D chromosomes. The primer sequences for the SSR markers were obtained from the GrainGenes website (http://wheat.pw.usda.gov/cgi-bin/graingenes/browse.cgi?class=marker) and are listed in Supplementary Table 1.

DArTseq™ analysis of F_2 progeny

Thirty F_2 seeds of each cross were planted in individual 200-mm-diameter plastic pots with Searle's® potting mixture in the glasshouse. DNA was extracted from the leaves of 3-week-old leaves using the CTAB method (Saghai-Maroof et al. 1984). The DNA quality was determined by gel electrophoresis using a 1% agarose gel and quantified with an Implen Nanophotometer (Integrated Sciences, Sydney, Australia). The DNA was normalised to 50 ng/μL and sent to DArT P/L. (Canberra, Australia) for DArTseq™ analysis. A hexaploid bread wheat array was used as the marker source and the sequencing was performed using the Illumina HiSeq 2500 platform and scored as 1 and 0 based on the presence or absence of unique A, B and D chromosomal sequences, respectively.

D genome analysis of F_3 progeny

Based on the results of the DArTseqTM analysis, six F_2 lines with known D chromosome content were chosen from each cross and studied in the subsequent F_3 generation. Five randomly chosen F_3 seeds from each of the six F_2 lines were planted in individual 200-mm-diameter plastic pots in the glasshouse. DNA was extracted from the leaf samples of 30 individual F_3 lines of each population (hexaploid/tetraploid and tetraploid/hexaploid) using a Wizard Genomic DNA Purification Kit (Promega Corporation). SSR markers listed in Supplementary Table 1 were used to determine the D chromosome content of the F_3 generation.

Statistical analysis

A pairwise *t* test was conducted to assess the differences in proportion of A and B chromosomes inherited from the tetraploid durum parent in the two crosses. Chi-square goodness of fit test was performed to assess the quality of DArTseqTM markers. The relationship between the inheritance of the A and B genomic sequence from the durum parent and the retention of D chromosomes were calculated using Spearman's correlation with the IBM SPSS statistical package version 22 (IBM-SPSS Inc., Chicago IL). Graphs were drawn using Prism Software version 5.01 (Graph Pad Software Inc., San Diego, CA).

Results

Quality control of DArTseq™ markers

A total of 7571 sequences were generated across the whole wheat genome using the DArTseq™ genotyping system. The average call rate for each sequence was 92.3%. Only sequences that were polymorphic between the two parents were used. Furthermore, sequences that

had missing values in more than 20 of the 60 samples were removed. Chi-square (χ^2) tests were conducted and sequences with segregation distortion ($P \geq 0.01$) were removed. The final number of sequences used in the analyses was 1580 across the A, B and D chromosomes (Supplementary Table 2). The number of polymorphic sequences generated for chromosomes A and B was 853, of which 562 sequences (66%) showed a positive signal on the hexaploid parent.

Proportion of chromosomes A and B inherited from the durum parent in the F_2 generation

The proportion of A and B durum-derived alleles inherited in 30 F_2 lines of each hexaploid/tetraploid and tetraploid/hexaploid cross was assessed with 853 DArT™ sequences (Supplementary Table 2). The overall mean proportion of chromosomes A and B alleles inherited from durum in the hexaploid/tetraploid cross was 45%. Progeny of the tetraploid/hexaploid cross retained 53 and 50% A and B durum parent alleles, respectively, mean values which were significantly different ($P = 0.049$) from the reciprocal cross (Supplementary Fig. 1). The pairwise *t* test indicated significant differences in the proportion of durum alleles inherited between the crosses in individual chromosomes 2B, 3A, 3B ($P < 0.01$) and 4A ($P = 0.043$) (Fig. 1). With the exception of chromosome 4A, these chromosomes possessed a higher percentage of durum alleles in the F_2 progeny from the tetraploid/hexaploid cross. In the most extreme example, the maximum proportion of durum chromosome 3A inherited in the hexaploid/tetraploid cross was only 30% while in the tetraploid/hexaploid cross this proportion was 87% (Supplementary Table 3). Individual F_2 lines had varying proportions of durum alleles ranging from 37 to 55% in the hexaploid/tetraploid cross and 35 to 62% in the tetraploid/hexaploid cross (Supplementary Table 3).

D genome retention in the F_2 generation

Analysis of 727 DArT sequences across all seven unique D chromosomes in 30 F_2 lines of both populations indicated a significant difference ($P = 0.003$) in the number of unique D chromosomes retained in each of these crosses. The hexaploid/tetraploid and tetraploid/hexaploid crosses retained an average of 1.83 and 2.83 D chromosomes per F_2 line, respectively. Of the 30 F_2 progeny from the hexaploid/tetraploid cross, 10 lines had lost both copies of all seven D chromosomes whereas only one line (3.3%) retained at least one copy of all seven D chromosomes (Fig. 2). In the reciprocal cross, five out of 30 lines (16.7%) lost all D chromosomes whereas three lines (10%) retained at least one copy of all seven D chromosomes. The remaining lines (73.4%) retained between one and six unique D chromosomes (Supplementary Fig. 2).

The identity of unique D genome chromosomes retained in both populations is given in Table 1. In both the LRC2010-150/WID802 and WID802/LRC2010-150 populations, chromosomes 4D (15 and 13 lines, respectively), 6D (11 and 12 lines, respectively) and 7D (10 and 18 lines, respectively) were retained in higher numbers than the other D chromosomes. Twenty-five out of 30 lines had lost the 3D chromosome in both populations. The remaining 1D, 2D and 5D chromosomes were more abundant in the tetraploid/hexaploid cross (Table 1). Complete loss of the long arms of chromosome 1D and 4D was observed in two individual lines (line LRC/WID-5 and LRC/WID-12, respectively; Supplementary Table 4) in the hexaploid/tetraploid cross, and complete loss of the short arm of chromosome 4D was observed in one line in the tetraploid/hexaploid cross (line WID/LRC-22; Supplementary Table 4).

Relationship between retention of durum-derived genomic material and retention of D chromosomes in F_2 lines

Analyses based on Spearman's correlation co-efficient between hexaploid/tetraploid (Fig. 3a) and tetraploid/hexaploid cross (Fig. 3b) indicated that there was a strong negative correlation ($r = -0.81$, $P < 0.001$) and ($r = -0.80$, $P < 0.001$), respectively, between the proportion of durum-derived A and B genome present in an F_2 individual and the number of D chromosomes retained. Thus, lines with a higher percentage of durum-derived segments generally retained fewer D chromosomes.

Retention of D chromosomes in the F_3 generation

Based on our analysis of the F_2 generation, we selected six lines that contained at least one copy of 1, 2, 3, 4 or 7 unique D chromosomes (as indicated in Fig. 4) from each reciprocal cross to evaluate the F_3 generation. Lines with a similar range of retained D chromosomes were selected for both crosses. The numbers of D chromosomes present in the F_3 generation were examined with a set of SSR markers (Supplementary Table 1).

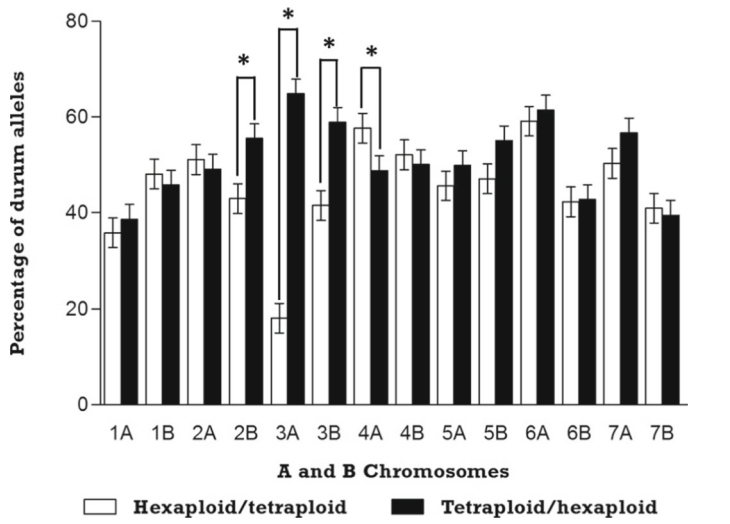


Fig. 1 Average percentage of retention of durum genomic material in each A and B chromosome in 30 F_2 lines from each cross. (*Asterisk*) Pairwise *t* test indicates significant differences for 2A, 3A, 3B ($P < 0.01$) and 4A ($P = 0.043$)

Significant differences were observed in the hexaploid/tetraploid and tetraploid/hexaploid crosses in the number of D chromosomes retained in the F_3 generations (Fig. 4). The reciprocal tetraploid/hexaploid cross resulted in the loss of a significant number of D chromosomes ($P = 0.035$) when compared to the hexaploid/tetraploid cross. The F_3 lines from the hexaploid/tetraploid cross retained 26% of the D chromosomes present in their F_2 parents, while the progeny from tetraploid/hexaploid F_2 lines retained only 15.2%. Even though both lines LRC/WID-29 and WID/LRC-1 retained at least one copy of each D chromosome in the F_2 generation, only the F_3 progeny of line LRC/WID-29 retained copies of all seven D chromosomes, whereas none of the five F_3

Fig. 2 Total number of unique D chromosomes retained in 30 F_2 lines from each cross. *Yellow-* and *red-coloured boxes* represent presence and absence of D chromosomes, respectively. A Hexaploid/tetraploid cross. B Tetraploid/hexaploid cross

Table 1 Retention of specific D chromosomes in 30 F_2 lines of each cross

Cross	NULL	1D	2D	3D	4D	5D	6D	7D
Hexaploid/tetraploid	10	5 + 1[a]	4	5	15 + 1[a]	5	11	10
Tetraploid/hexaploid	5	14	11	5	13 + 1[a]	11	12	18

[a] Lines containing only a partial copy of this chromosome

lines generated from WID/LRC-1 retained the full set. This result suggests that only the hexaploid/tetraploid F_2 line LRC/WID-29 retained stable pairs of all seven D chromosomes and possessed a total chromosome number of 42. In contrast, only chromosome 6D was present as a stable pair in the tetraploid/hexaploid line WID/LRC-1 (Fig. 4).

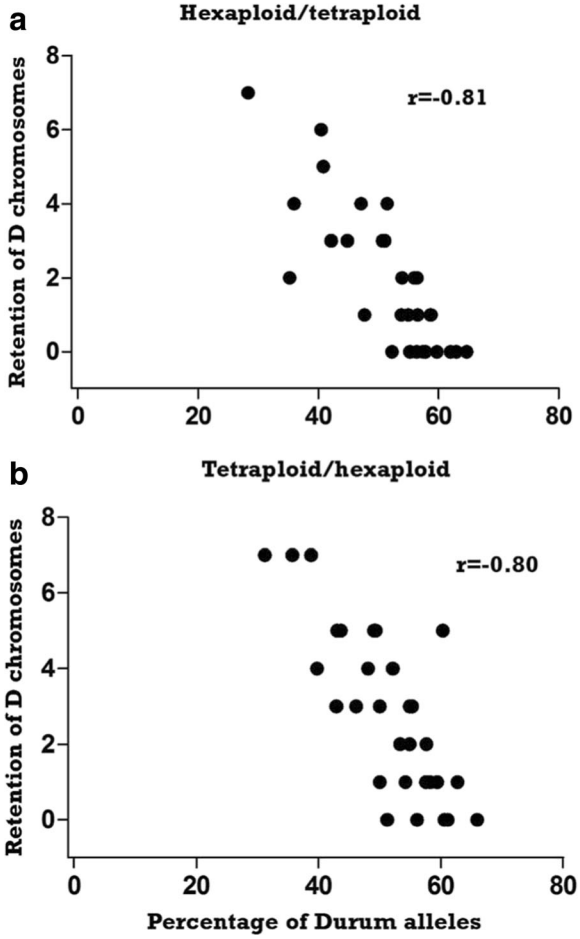


Fig. 3 Correlation between the percentage of durum alleles and retention of the D genome in 30 F_2 lines of the hexaploid/tetraploid cross (a) and the tetraploid/hexaploid cross (b)

Discussion

This study is the first to directly compare the inheritance of parental genomic material in a pair of reciprocal interspecific crosses between the same tetraploid durum and hexaploid bread wheat parents. Crossing of the two parents chosen for this work produced more viable progeny than did crossing of several other parental pairs evaluated regardless of the direction of the crosses. Previous studies investigating interspecific hybridisation of different ploidy level wheat species were also hampered by low seed set and even when seed set occurred, successful germination and hybrid seedling establishment were not always achieved (Bhagyalakshmi et al. 2008).

Significant differences were observed in the overall proportion of A and B genomes inherited from the durum parent in the pair of reciprocal crosses examined (Supplementary Fig. 1). Significant differences were also observed between the two populations with respect to individual chromosomes 2B, 3A, 3B and 4A, which, with the exception of chromosome 4A, retained a greater percentage of durum alleles in the hexaploid/tetraploid, cross (Fig. 1). A recent study by Kalous et al. (2015) using tetraploid and hexaploid recombinant inbred lines (RIL) derived from hexaploid/tetraploid crosses identified positive alleles for grain weight on chromosome 3B inherited from the tetraploid parent. A durum line carrying these alleles for grain weight on chromosome 3A, in which there is segregation distortion towards retention of that chromosome segment in interspecific crosses to bread wheats, would be an excellent candidate source for introgression of this trait into hexaploid lines.

DArTseq™-based analyses of the D genome inheritance in the F_2 populations indicated a significant difference in the average number of D chromosomes retained per F_2 line, with lines resulting from the tetraploid/hexaploid cross retaining a greater mean number of D chromosomes than those resulting from the hexaploid/tetraploid cross. In the current study, the identity of D chromosomes most frequently retained varied

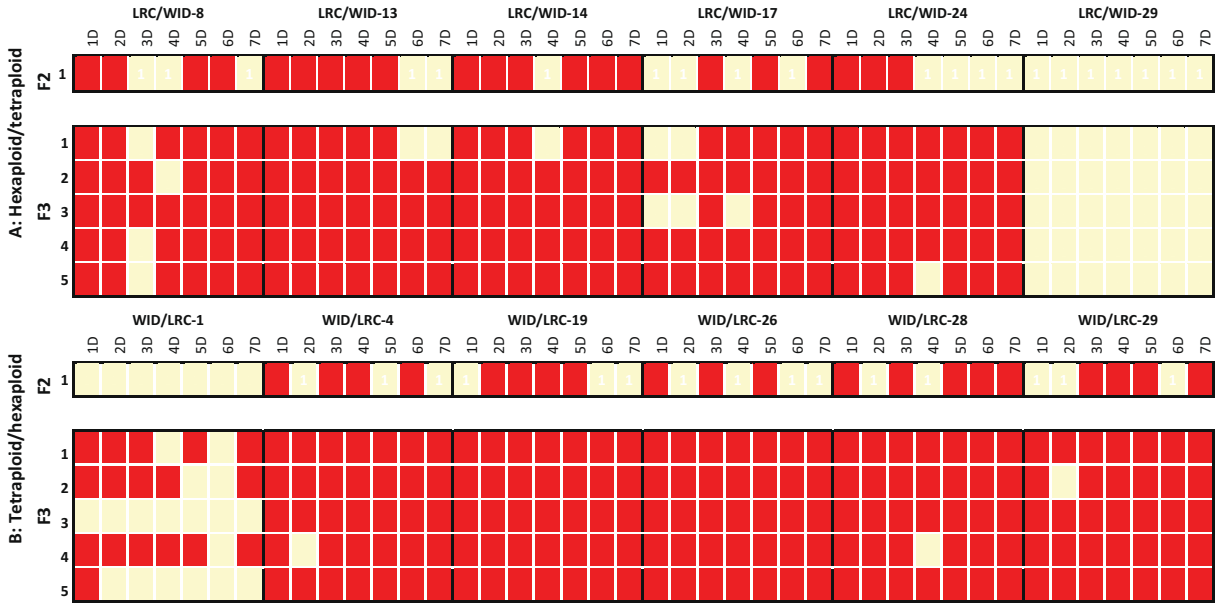


Fig. 4 Retention of the D genome in the F_2 and F_3 generations. D chromosomes present in the six selected F_2 lines are given followed by the five progeny of each F_2 line. Each *column* represents a different D genome chromosome. *Yellow* and *red boxes* represent presence and absence of D chromosomes, respectively. **A** Hexaploid/tetraploid. **B** Tetraploid/hexaploid cross

somewhat between the reciprocal crosses, with 4D, 6D and 7D being observed more frequently in both populations, and 3D less frequently. However, for 1D, 2D and 5D, their relative retention varied between the populations. These results indicating that particular D chromosomes are more stably retained require further examination of other crosses and their reciprocals. In an earlier study, Martin et al. (2011) assessed D genome retention across eight populations developed from five hexaploid/tetraploid crosses. In the five populations which retained significant quantities of D genome, the subset of D chromosomes present appeared to be random.

With respect to the partial D chromosomes identified in the study, we identified complete loss of entire chromosome arm in three out of 60 F_2 lines examined. Unfortunately, these lines did not set seed and further cytological investigation of F_3 progeny could not take place. A number of other studies have identified partial D chromosomes and have through the application of cytological MCFISH found that these are translocations (Eberhard et al. 2010; Martin et al. 2011). It is a general tendency for alien introduced univalent chromosomes to undergo centric breakage-fusion and lead to chromosomal translocations/deletions (Sharma and Gill 1983).

Even though molecular markers help to identify the presence or absence of unique D chromosomes, they fail to identify if one or two copies of a particular locus are present. Using only molecular markers, it is also difficult to identify the telocentric or translocation chromosomes in the presence of a complete homologous pair. Cytological techniques such as MCFISH and GISH (genomic in situ hybridisation) can be used to determine the number of copies of chromosomes that are present (Zhang and Friebe 2009).

A strong negative correlation was observed in both F_2 populations between the retention of D chromosomes and the proportion of A and B genomes inherited from the durum parent. Martin et al. (2011) also observed that hexaploid/tetraploid derived populations with the lowest numbers of D chromosomes had higher percentages of durum alleles. These results suggest that the number of D chromosomes retained in an F_2 population can be used to predict whether an interspecific cross would be more suitable for the introgression of traits from durum wheat into bread wheat or from bread wheat into durum wheat.

In the F_3 generation further elimination of D chromosomes occurred in both populations, particularly in the population derived from the tetraploid/hexaploid cross. The stable inheritance of a particular D chromosome in subsequent generations is dependent on the copy number present. For example, the F_2 lines LRC/WID-29 and WID/LRC-1 both retained at

least a single copy of every D chromosome. However in the F_3 generation, only one of the five individuals derived from the line WID/LRC-1 retained all seven unique chromosomes and only 6D was retained in all five lines, implying this was the only D chromosome present as a pair in the F_2 generation. In contrast, all five F_3 progeny from the hexaploid/tetraploid cross (LRC/WID-29) retained copies of all seven D chromosomes in the F_3 generation. This suggests that this F_2 line possessed a pair of each copy of D chromosome and therefore a stable hexaploid chromosome number of 42. The rapid elimination of D chromosomes in generations derived from this particular pair of reciprocal crosses may be due to the pedigree of the parent LRC2010-150 (GW95-703*C15/Lang). GW95-70*C15 is a doubled haploid line derived from the synthetic line CPI133814. Crosses of this synthetic hexaploid wheat cultivar with the durum variety EGA Bellaroi, when compared to other hexaploids crossed with EGA Bellaroi, rapidly lost D chromosomes within two generations (Martin et al. 2011).

We undertook this study to determine whether differences in chromosome constitution depended on the direction of the interploidy cross. Differences in the proportion of A and B chromosomes inherited were not significant but lines resulting from the hexaploid/tetraploid cross on average retained fewer unique D chromosomes in the F_2 generation than lines from the tetraploid/hexaploid cross. Whether this is a general phenomenon across similar interspecific crosses remains to be investigated. However, lines with none or all seven D chromosomes were generated in both reciprocal crosses suggesting either population could be used to produce tetraploid or hexaploid lines. Given that hexaploid/tetraploid crosses are generally more successful in producing viable progeny (Ramsey and Schemske 1998; Kalous et al. 2015), these results support the use of hexaploid/tetraploid crosses in the first instance. Reciprocals of other interspecific crosses will need to be examined to confirm this conclusion.

Acknowledgements We thank Dr. Cassy Percy for providing us with hexaploid bread wheat LRC2010-150 seed. Part of the work was financially supported by the Grains Research and Development Corporation, Australia. The authors would like to thank Dr. Kathleen L. Farquharson (Plant Editors) for her critical comments on this manuscript. Sriram Padmanaban acknowledges the support of an Australian Postgraduate Award.

References

Bhagyalakshmi K, Vinod KK, Kumar M, Arumugachamy S, Prabhakaran A, Raveendran TS (2008) Interspecific hybrids from wild x cultivated *Triticum* crosses—a study on the cytological behaviour and molecular relations. J Crop Sci Biotech 11:257–262

Eberhard FS, Zhang P, Lehmensiek A, Hare RA, Simpfendorfer S, Sutherland MW (2010) Chromosome composition of an F_2 *Triticum aestivum* X *T. turgidum* spp. *durum* cross analysed by DArT markers and MCFISH. Crop Pasture Sci 61:619–624

Gilbert J, Procunier J, Aung T (2000) Influence of the D genome in conferring resistance to Fusarium head blight in spring wheat. Euphytica 114:181–186

Gupta PK, Varshney R (2000) The development and use of microsatellite markers for genetic analysis and plant breeding with emphasis on bread wheat. Euphytica 113:163–185

Kalous J, Martin J, Sherman J, Heo H-Y, Blake N, Lanning S, Eckhoff J, Chao S, Akhunov E, Talbert L (2015) Impact of the D genome and quantitative trait loci on quantitative traits in a spring durum by spring bread wheat cross. Theor Appl Genet 128:1799–1811

Kihara H (1925) Weitere Untersuchungen über die pentaploiden *Triticum*-Bastarde. I Jpn J Bot 2:299–305

Kihara H (1982) Wheat studies retrospect and prospects, Kodansha Ltd, Tokyo

Kopecký D, Bartoš J, Lukaszewski AJ, Baird JH, Černoch V, Kölliker R, Rognli OA, Blois H, Caig V, Lübberstedt T (2009) Development and mapping of DArT markers within the *Festuca-Lolium* complex. BMC Genomics 10:473

Lanning SP, Blake NK, Sherman JD, Talbert LE (2008) Variable production of tetraploid and hexaploid progeny lines from spring wheat by durum wheat crosses. Crop Sci 48:199–202

Martin A, Simpfendorfer S, Hare R, Sutherland M (2013) Introgression of hexaploid sources of crown rot resistance into durum wheat. Euphytica 192:463–470

Martin A, Simpfendorfer S, Hare RA, Eberhard FS, Sutherland MW (2011) Retention of D genome chromosomes in pentaploid wheat crosses. Heredity 107:315–319

Padmanaban S, Zhang P, Hare RA, Sutherland MW, Martin A (2017) Pentaploid wheat hybrids: applications, characterisation, and challenges. Front Plant Sci 8:358. doi:10.3389/fpls.2017.00358

Ramsey J, Schemske DW (1998) Pathways, mechanisms, and rates of polyploid formation in flowering plants. Annu Rev Ecol Syst 29:467–501

Ren R, Ray R, Li P, Xu J, Zhang M, Liu G, Yao X, Kilian A, Yang X (2015) Construction of a high-density DArTseq SNP-based genetic map and identification of genomic regions with segregation distortion in a genetic population derived from a cross between feral and cultivated-type watermelon. Mol Gen Genomics 290:1457–1470

Riley R, Chapman V (1967) The inheritance in wheat of crossability with rye. Genet Res 9:259–267

Saghai-Maroof MA, Soliman KM, Jorgensen RA, Allard R (1984) Ribosomal DNA spacer-length polymorphisms in barley: Mendelian inheritance, chromosomal location, and population dynamics. Proc Natl Acad Sci U S A 81:8014–8018

Sharma HC, Gill BS (1983) Current status of wide hybridization in wheat. Euphytica 32:17–31

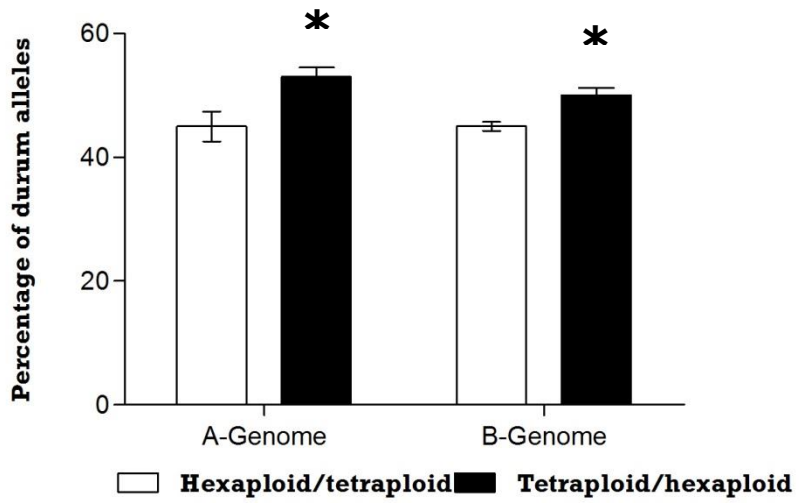
Somers DJ, Isaac P, Edwards K (2004) A high-density microsatellite consensus map for bread wheat (*Triticum aestivum* L.) Theor Appl Genet 109:1105–1114

Wang H, Liu D, Yan Z, Wei Y, Zheng Y (2005) Cytological characteristics of F_2 hybrids between *Triticum aestivum* L. and *T. durum* Desf. with reference to wheat breeding. J Appl Genet 46:365–369

Xu L, Wang M, Cheng P, Kang Z, Hulbert S, Chen X (2013) Molecular mapping of *Yr53*, a new gene for stripe rust resistance in durum wheat accession PI 480148 and its transfer to common wheat. Theor Appl Genet 126:523–533

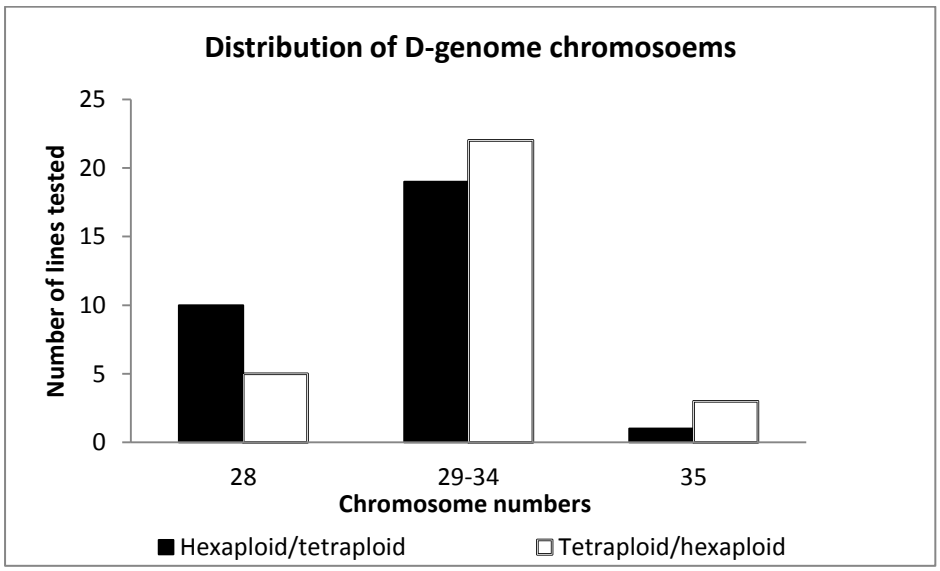
Zhang P, Friebe B (2009) FISH on plant chromosomes. In: Liehr T (ed) Fluorescence in situ hybridization (FISH)—application guide. Springer, Berlin Heidelberg, Berlin, Heidelberg, pp 365–394

Supplementary Material:



* t-test indicates significant difference, P=0.0493

Supplementary Figure-1 percentage of A and B genome alleles inherited



Supplementary Figure-2 Distribution frequency of D-genome chromosomes in 60 F_2 progeny

Supplementary Table 1. SSR markers used to identify the seven unique D chromosomes

Chromosome	1D	2D	3D	4D	5D	6D	7D
Short arm	wmc432	wmc603	cfd141	wmc457	cfd18	cfd49	cfd46
Centromere	cfd82	wmc18	barc42	barc334	cfd78	cfd287	cfd14
Long arm	cfd32	cfd233	barc270	cfd84	barc110	cfd95	barc235

wmc432	Forward 5'ATGACACCAGATCAGCAC3' Reverse 5'AATATTGGCATGATTACACA3'
cfd82	Forward 5' GCTGATGCTGCTGTAAGTGC 3' Reverse 5' TGAAGAATACAATGGCAGCAA 3'
cfd32	Forward 5' CAACACAACCACAATTTCCG 3' Reverse 5' CTCAGGGAGGTCATGCAGAG 3'
wmc603	Forward 5'ACAAACGGTGACAATGCAAGGA3' Reverse 5'CGCCTCTCTCGTAAGCCTCAAC3'
wmc18	Forward 5'TGGGGCTTGGATCACGTCATT3' Reverse5'AGCCATGGACATGGTGTCCTTC3'
cfd233	Forward 5' GAATTTTTGGTGGCCTGTGT 3' Reverse 5' ATCACTGCACCGACTTTTGG 3'
cfd141	Forward 5' CGTAAAGATCCGAGAGGGTG 3' Reverse 5' TCCGAGGTGCTACCTACCAG 3'
barc42	Forward 5' GCGACTCCTACTGTTGATAGTTC 3' Reverse 5' GCGTTCTTTTATTACTCATTTTGCAT 3'
barc270	Forward 5' GCGCATTGTGACAGGTGAAC 3' Reverse 5' GGAGGGAGTACTTGGTTATTAGGG T 3'
wmc457	Forward 5' CTTCCATGAATCAAAGCAGCAC3' Reverse 5' CATCCATGGCAGAAACAATAGC3'
barc334	Forward 5' ATCCGCGTGTCAAACTTCTTCC 3' Reverse5' GGGCTGGCTGGGCTAAATG 3'
cfd84	Forward 5' GTTGCCTCGGTGTCGTTTAT 3' Reverse 5' TCCTCGAGGTCCAAAACATC 3'
cfd18	Forward 5' CATCCAACAGCACCAAGAGA 3' Reverse 5' GCTACTACTATTTCATTGCGACCA 3'
cfd78	Forward 5' ATGAAATCCTTGCCCTCAGA 3' Reverse 5' TGAGATCATCGCCAATCAGA 3'
barc110	Forward 5' CCCGAACAATGGCTTTGGTGTCGTAAT 3' Reverse 5' CATGGTGACGGCAAGTGTGAGGT 3'
cfd49	Forward 5' TGAGTTCTTCTGGTGAGGCA 3' Reverse 5' GAATCGGTTCACAAGGGAAA 3'
cfd287	Forward 5' TCAAGAAGATGCGTTCATGC 3' Reverse 5' GGGAGCTTTCCCTAGTGCTT 3'
cfd95	Forward 5' AATCCTGACTTTAAAGCCTTTCC 3' Reverse 5' CATCTGTATGATATTTTGGAGGTCA 3'
cfd46	Forward 5' TGGTGGTATAGTCGTTGGAGC 3' Reverse 5' CCACACACACACACCATCAA 3'
cfd14	Forward 5' CCACCGGCCAGAGTAGTATT 3' Reverse 5' TCCTGGTCTAACAACGAGAAGA 3'
barc235	Forward 5' GCGCTCACCCTCCTACACTTCCTA 3' Reverse 5' GCGCAAGTCTGTCAAAGCCTAA 3'
cfa2278	Forward 5' GCCTCTGCAAGTCTTTACCG 3' Reverse 5' AAGTCGGCCATCTTCTTCCT 3'
wmc120	Forward 5' GGAGATGAGAAGGGGGTCAGGA Reverse 5' CCAGGAGACCAGGTTGCAGAAG
gwm408	Forward 5' TCGATTTATTTGGGCCACTG 3' Reverse 5' GTATAATTCGTTCACAGCACGC 3'

Supplementary Table 2. Number of DArTseq™ markers for each unique A, B and D chromosome

Chromosome number	Genome		
	A	B	D
1	52	78	99
2	69	82	146
3	58	97	125
4	87	34	107
5	22	57	84
6	47	52	67
7	58	60	99
Total number of markers	**393**	**460**	**727**
Average number of markers	**58**	**66**	**104**

Supplementary Table 3. Percentage of durum parent alleles inherited by individual chromosomes in each cross.

F_2 lines

Chromosomes	LRC/WID-1	LRC/WID-10	LRC/WID-11	LRC/WID-12	LRC/WID-13	LRC/WID-14	LRC/WID-15	LRC/WID-16	LRC/WID-17	LRC/WID-18	LRC/WID-19	LRC/WID-2	LRC/WID-20	LRC/WID-21	LRC/WID-22	LRC/WID-23	LRC/WID-24	LRC/WID-25	LRC/WID-26	LRC/WID-27	LRC/WID-28
1A	47.3	30.2	45.6	72.4	21.9	49.1	39.6	32.8	46.7	28.7	22.1	23.5	52.9	21.9	20.7	37.3	34.4	17.8	30.9	29.4	22.9
1B	49.1	60.6	52.1	76.3	57.8	36.3	27.1	42.5	17.9	39.1	38.4	58.7	67.4	40.7	38.8	57.2	60.9	42.3	39.9	47.8	37
2A	51.8	38.2	61.5	64.5	45	70.3	48.8	45.4	48.5	54.2	40.6	30.5	48	36.6	44.2	40.2	42.2	60.5	62.2	48.4	56.7
2B	40	40.6	46.5	18.7	43.4	55	17.8	24.3	77.1	42.4	72.2	41.5	32.7	50.4	36.8	39.5	36.5	24.5	54.1	45.2	50.7
3A	11.1	16.7	5.71	15.7	14.3	13.6	24.3	15.2	22.1	8.57	26.5	14.1	16.2	14.3	25	17.1	15.3	27.3	10.6	6.06	22.7
3B	15	11	36.8	58.1	34.8	34.3	50.1	40.6	20.6	40.4	45.8	25.4	31.8	44.9	84.9	45.9	18.7	57.2	14.7	18.1	62.5
4A	43.8	54.8	20.3	33	67.6	49	34.8	76.6	31.7	81.6	96.1	79.9	53.4	86	43.5	65.5	68.4	28	73.6	87.9	55.7
4B	52.4	51.5	55.2	42.1	41.1	42	51.2	45.1	48.4	55	53.1	60.6	53.3	58.6	67	54.4	60	61.6	50.6	59	53.6
5A	51	53.3	78.6	41.2	25	55.8	68.1	34.1	56	33.8	46.7	39.3	26.7	28.6	38.6	46.7	58.3	48.1	57.6	64.3	29.8
5B	44.1	37.2	39.6	57.8	46	47	63.3	37.3	33.7	70.8	46.2	35.4	40.3	62.9	50.4	49.6	19.7	30.5	42.5	50.8	67
6A	91.5	59.8	62.4	70.4	50	65.4	93.7	37.5	81.4	17.6	47.5	53.5	56.4	68.6	57.3	34.5	40.3	36.6	85.4	57.7	77.5
6B	49.1	8.89	66.9	56.5	42.7	15.3	33.5	28.9	27.4	70.7	48.1	61.4	77.1	38.1	33.9	32.8	27.3	27.5	42.2	47.6	4.68
7A	23.4	49.4	74	38.7	94.1	45.6	34.3	75.9	78.8	50.1	85.2	52	67.7	13.9	31.2	85.1	22.2	51	69.5	47.4	45.8
7B	23.8	55.6	43.9	48.7	36.1	34.3	58.3	37.5	38	53.7	17.1	23.8	66.7	21.5	19.5	87.1	20.2	27.9	41.6	89.8	63.8
Line average	42.4	40.6	49.2	49.6	44.3	43.8	46.1	41	44.9	46.2	49	42.8	49.3	41.9	42.3	49.5	37.5	38.6	48.2	50	46.4

F_2 lines

Chromosomes	WID/LRC-1	WID/LRC10	WID/LRC-11	WID/LRC-12	WID/LRC-13	WID/LRC-14	WID/LRC-15	WID/LRC-16	WID/LRC-17	WID/LRC-18	WID/LRC-19	WID/LRC-2	WID/LRC-20	WID/LRC-21	WID/LRC-22	WID/LRC-23	WID/LRC-24	WID/LRC-25	WID/LRC-26	WID/LRC-27	WID/LRC-28
1A	60.2	23.5	43.5	27.1	42.8	25.8	39.2	69.5	22.7	7.58	24.2	50.3	42.3	24.3	44.2	26.5	24.1	64.2	79.8	41.7	70.1
1B	38.2	36.8	49.5	34.9	65.8	48.8	38.9	41.6	31.7	56.2	32.4	61.2	27.5	39.4	54	46.5	47.3	40.1	31.2	47.6	62.1
2A	70.2	60.7	51.4	44.3	32.8	65.6	35.1	67.7	56.5	41.3	62.8	65.5	42.5	58.6	33.6	61.4	54.6	39	49.5	48.3	27.4
2B	40.7	89	46.5	47.4	70.1	71.8	24.6	65.3	42.4	37.1	58.9	59.5	66.9	55.7	32.7	79.4	53.8	21.2	47.7	72.9	36.2
3A	52.2	60.7	49	72.5	75	79.5	67.9	61.5	73.9	25.1	58.4	79.1	64.1	56.7	70.9	87.3	48.2	28.2	78.6	71.7	86.2
3B	17.3	55.9	37.5	45.2	56.9	70.4	65.2	34.9	50.4	37	36.1	70.7	57.1	71	69.4	70.5	36.5	31.2	67.2	77.2	79.2
4A	48.9	48.3	16.9	37.8	82.6	24.9	13.9	21.4	22.6	50.9	51.7	39.4	71.6	39.8	38.2	64.3	79.3	17.8	56.9	84	9.94
4B	36	44.1	58.4	60.7	52.8	50.4	48.3	52.1	45	49.8	41.3	61.7	41.7	57.4	52.8	57.8	46.9	42.7	55.6	58	53.7
5A	21.4	50	51.8	61.9	45.8	50.5	53.6	38.3	51.4	7.14	48.8	75	60.9	53.6	11.9	61.9	61.7	10	51	60.7	38.1
5B	49.6	51.9	62.7	74.3	62	44.4	34.3	83.5	28	55.4	42	67.1	23	42.9	49.5	63.7	62.3	53.7	72	46.7	58.9
6A	73	87.1	44.9	87.5	76.1	47	89.2	64.6	33.5	85	64.7	61.9	23.3	53.6	68.5	29.5	80	20.5	80.9	36.7	70.6
6B	41.7	32.5	24.8	52.3	83.9	47.6	40.7	31.5	56.7	55	15.3	27.6	54.2	31	58.7	25.7	42.7	36.4	70.5	38.9	51.7
7A	49.8	48.8	43.2	69.5	93.3	30.6	59.3	43	69.2	20.7	64.6	57.7	56.9	92.2	52.4	98.5	38.5	46	60.7	59.4	59.4
7B	31.9	51.3	55.9	45.6	31.7	21.3	40.2	20.4	21.3	15.3	91.3	22.2	28.1	24.6	53.6	59.3	32.3	39.5	20	16.3	32.4
Line average	45.1	52.9	45.4	54.4	62.3	48.5	46.5	49.7	43.2	38.8	49.5	57.1	47.1	50	49.3	59.4	50.6	35	58.7	54.3	52.6

Lines which retained the maximum and minimum average % of durum alleles are highlighted in yellow and red, respectively.

LRC/WID-29	LRC/WID-3	LRC/WID-30	LRC/WID-4	LRC/WID-5	LRC/WID-6	LRC/WID-7	LRC/WID-8	LRC/WID-9	Minimum	Maximum	Average
22.8	37.39	47.44	18.57	23.5	59.3	77.5	27	32.4	17.8	77.45	35.8
36.6	72.92	75.29	34.56	23.6	77.8	59.6	30.2	45	17.9	77.77	48.1
26.6	65.57	58.61	75.8	52.1	41.7	40.3	51.5	82.7	26.6	82.74	51.1
54.1	48.21	35.4	26.36	48.1	37	63.5	38.2	47.1	17.8	77.14	42.9
28.1	27.78	22.06	5.56	18.1	20	30.3	27.3	19.4	5.56	30.3	18
48	45.11	53.98	48.31	48.7	26.4	81.7	60.3	41.5	11	84.9	41.5
72.3	90.36	65.33	40.97	42.7	66.6	56.1	39.7	24.7	20.3	96.05	57.7
50	59.38	44.1	41.39	48.3	51.7	55.2	48	49.6	41.1	67.02	52.1
25	37.14	68.33	50	61.4	50	17.9	29.8	47.6	17.9	78.57	45.6
48.6	48.65	50.89	42.17	43.4	48	32.9	50.3	75.3	19.7	75.25	47.1
38.1	74.91	97.73	33.43	73.7	45.6	38	64	62.1	17.6	97.73	59.1
30.4	47.02	75.99	37.14	47.9	34.2	71.7	46.4	37	4.68	77.14	42.3
48.2	45.32	52.68	9.46	46.7	49.6	25.4	42.9	53	9.46	94.14	50.3
16.9	66.92	19.57	53.72	72.9	11.3	12.5	43.6	21.8	11.3	89.81	40.9
39	54.76	54.81	36.96	46.5	44.2	47.3	42.8	45.7	37	54.81	45.2

WID/LRC-29	WID/LRC-3	WID/LRC-30	WID/LRC-4	WID/LRC-5	WID/LRC-6	WID/LRC-7	WID/LRC-8	WID/LRC-9	Minimum	Maximum	Average
25.9	38.53	25.67	25.76	80.9	30.1	17.7	22.8	38	7.58	80.89	38.6
54.1	53.48	54.73	39.82	36.8	66.8	41.7	39.2	55.1	27.5	66.83	45.8
36.3	39.44	53.61	50.39	45.2	43.9	61.4	29.3	45.2	27.4	70.15	49.1
29.9	66.94	67.17	44.49	85.2	60.3	71.1	50	69.7	21.2	88.97	55.5
48.1	66.27	71.66	71.21	69.7	58.3	64.8	64.2	84	25.1	87.3	64.8
73.3	62.16	41.88	77.61	74.8	81.7	83.7	84.9	49.6	17.3	84.9	58.9
79.8	85.95	30.5	69.84	49.2	43.9	81.1	37.5	65.6	9.94	85.95	48.8
49.3	44.79	37.5	60	60.8	52.3	40.6	35.9	53.1	35.9	61.67	50
71.9	69.29	50.95	56.92	72.1	47.7	35.7	54.2	71.4	7.14	75	49.9
65.9	75.67	54.7	54.51	56.1	47	64.3	63.4	42.3	23	83.46	55.1
6.55	78.1	68.18	100	55.4	47.7	90.9	73.3	44.9	6.55	100	61.4
11.3	60.88	56.09	30.66	45.7	43.6	55.7	27.2	32.1	11.3	83.88	42.8
36.6	25	53.55	47.13	88.8	80.7	74	40.1	40	20.7	98.48	56.7
59.2	28.44	71.28	55.77	22.6	36	32.2	39.9	84.7	15.3	91.25	39.5
46.3	56.78	52.68	56.01	60.2	52.9	58.2	47.3	55.4	35	62.26	51.2

Chapter 5

Association between presence of *Triticum timopheevii* introgression and D-genome retention in hexaploid/tetraploid wheat crosses.

The third study aims to understand how hexaploid bread wheat cultivars with an introgressed 2G segment inherit when combining with tetraploid durum wheat cultivars. Unique sets of hexaploid/tetraploid crosses were produced by choosing a maternal Sunguard parent with 2G introgression and crossed with current Australian durum wheat cultivars such as Caparoi, Hyperno, and WID802 as paternal parents. The objectives of the study were to examine (i) the degree of inheritance of the 2G introgressed segment in F_2 progenies of different crosses; (ii) differences in the retention of D-genome chromosomes between the crosses; (iii) the relationship of the introgressed 2G chromosomal segment to the retention of D-genome chromosomes in F_2 and F_4 progenies. The content of this chapter is published in the journal "Crop and Pasture Science" and details are below.

Padmanaban S, Zhang P, Sutherland MW and Martin A (2018) "Association between presence of *Triticum timopheevii* introgression and D-genome retention in hexaploid/tetraploid wheat crosses". Molecular Breeding 38:84. doi: 10.1007/s11032-018-0838-6

Note: Supplementary data associated with the chapter are attached along with the article.

Mol Breeding (2018) 38:84
https://doi.org/10.1007/s11032-018-0838-6

Association between presence of *Triticum timopheevii* introgression and D-genome retention in hexaploid/tetraploid wheat crosses

Sriram Padmanaban · Peng Zhang · Mark W. Sutherland · Anke Martin

Received: 15 February 2018 / Accepted: 28 May 2018

Abstract The 2G *Triticum timopheevii* introgression harbours genes for multiple disease resistance and quality traits in bread wheat. In order to transfer this segment from bread wheat into durum, the bread wheat line Sunguard, which carries this introgressed 2G segment was crossed with three tetraploid durum parents. A significant difference was observed in the segregation ratio of the 2G segment in the different crosses at the F_2 generation with two of the three populations indicating segregation distortion against the hexaploid 2G segment. In these populations, the presence of the 2G segment was strongly correlated with the presence of D-genome chromosomes. These results were confirmed in the F_4 generation of these populations. Six plants were identified in the F_4 generation, which had retained the introgressed 2G segment in a homozygous condition and did not have a complete D-genome set. Two of these lines only had two non-homologous D-genome chromosomes in the F_5 generation. Thus, the 2G segment and possibly other translocations can be transferred into durum wheat through hexaploid/tetraploid hybridisation.

Keywords 2G introgression · Inter-ploidy crosses · Wheat breeding · *T. timopheevii* 2G

Introduction

Discovering, developing and characterising novel sources of genetic variation for disease or pest resistances in wheat is a continuous process essential to maintaining crop health and sustaining productivity. Bread wheat (*Triticum aestivum* L.) and its related wheat species possess homologous or partly homologous genomes that harbour large beneficial allelic combinations for various disease resistances (Dundas et al. 2007). Several resistance genes that are effective against multiple pests and diseases have been introgressed into commercial bread wheat cultivars from wild relatives (Friebe et al. 1996; Molnár-Láng et al. 2015). There are a number of difficulties involved in the introgression of chromosomal segments from related and wild species into commercial wheat cultivars, such as genome compatibility and ploidy level. However, these difficulties can be overcome by selecting suitable breeding techniques and compatible genotypes (Jiang et al. 1993; Friebe et al. 1996).

A number of introgressions harbouring disease-resistance genes have been successfully deployed in bread wheat cultivars, and these have played a significant role in plant breeding for crop improvement and food security (Wulff and Moscou 2014). One example is the translocation of the tetraploid *T. timopheevii* (A^tA^tGG) segment on chromosome 2G to chromosome 2B in hexaploid wheat. This 2G introgression contains a number of

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s11032-018-0838-6) contains supplementary material, which is available to authorized users.

S. Padmanaban · M. W. Sutherland · A. Martin (✉)
Centre for Crop Health, University of Southern Queensland, Toowoomba, Queensland 4350, Australia
e-mail: anke.martin@usq.edu.au

P. Zhang
Plant Breeding Institute, The University of Sydney, Cobbitty, New South Wales 2570, Australia

Published online: 19 June 2018

desirable loci that confer resistance to stem rust (Olson et al. 2010; Bariana et al. 2001; Dundas et al. 2007), leaf rust (Leonova et al. 2007; Leonova et al. 2002), powdery mildew (Friebe et al. 1996; Tao et al. 2000), crown rot (Bovill et al. 2006) and black point (Lehmensiek et al. 2004). It also contains certain allelic combinations that are associated with improved grain quality characters, such as increased milling yield (Lehmensiek et al. 2006). A number of commercial Australian bread wheat varieties, including Sunco, Lang, Sunvale and Sunguard, possess this introgression (Friebe et al. 1996).

Alien introgressions have mainly been transferred from wild relatives into hexaploid wheat (Mago et al. 2005; Friebe et al. 1996). To our knowledge, only one study has reported on the transfer of an introgresssion from bread wheat into durum wheat (Martin et al. 2013). This study crossed Sunco with four different durum parents and found that the 2G introgression present in Sunco had not been transferred in two of the four crosses made. Detailed results with regard to the presence of 2G introgression in the remaining two populations were not presented and its presence in later generations was not investigated.

Developing pentaploid (AABBD, 5X) derived lines by crossing hexaploid (AABBDD, 6X) with tetraploid (AABB, 4X) wheat to transfer crown rot resistance into durum is one of the major foci of our research group (Eberhard et al. 2010; Martin et al. 2011; Martin et al. 2013; Padmanaban et al. 2017b). Our investigations have demonstrated wide variation between different pentaploid crosses both in the proportions of parental sequences inherited by the F_2 A and B genomes and in the number of D chromosomes retained by the F_2 generation (Padmanaban et al. 2017a).

This current study examines the transfer of the 2G segment from hexaploid Sunguard wheat, into a range of durum backgrounds. In particular, we have examined the proportion of F_2 progeny which inherit the 2G segment, the retention of D-genome chromosomes in each of the crosses and the relationship between retention of the 2G segment and the retention of D-genome chromosomes in subsequent generations.

Materials and methods

Plant materials

Spring bread wheat, Sunguard (Sun289E/Sr2Janz), was crossed with spring durum wheat cultivars Caparoi (LY2.6.3/930054), Hyperno (Kalka sister line/Tamaroi) and WID802 (Syrica-1/Yallaroi//Tamaroi/Lingzhi/Yallaroi*2///RAC875/Kalka//Tamaroi////Lingzhi/Yallaroi//Tamaroi///Lingzhi/Yallaroi). Hexaploid/tetraploid crosses were developed in the glasshouse during 2014 following traditional hand emasculation and pollination (Riley and Chapman 1967). The F_1 seeds from the pollinated heads were carefully collected and individually germinated in 24-well plates containing 2% water agar. One-week-old seedlings were transplanted to 200-mm-diameter plastic pots containing standard potting mixture (Searle's® certified premium grade, Searle Pty. Ltd., Brisbane). The seedlings were grown in the glasshouse and F_2 seeds were harvested at plant maturity.

DNA was extracted from individual 3-week-old F_1 seedlings using a Wizard Genomic DNA purification kit (Promega Corporation, Sydney, Australia) as per the manufacturer's instructions. Individually extracted F_1 DNA samples were subjected to marker analysis to confirm their heterozygosity using selected microsatellite markers (cfa2278 and gwm345 on chromosome 2B) that showed polymorphism between the parents.

Microsatellite characterisation for the inheritance of the 2G segment and D-genome retention of F_2 generation

Sixty randomly selected F_2 seedlings were raised from each of the Sunguard/Caparoi, Sunguard/Hyperno and Sunguard/WID802 crosses. DNA was extracted from the 180 individual F_2 plants and marker analysis was carried out to determine whether the 2G segment was present. Based on the consensus, molecular genetic map (Appels 2003) microsatellite primers on chromosome 2B that cover the complete introgressed 2G segment (cfa2278, gwm630, gwm319, wmc360, wmc441, and gwm501) were used. A set of 21 microsatellite primers (three for each of the seven non-homologous D chromosomes covering the long and short arms and the centromere region) were used to investigate the retention of D-genome chromosomes in the F_2 plants (Padmanaban et al. 2017a). The same markers were used to investigate the retention of D-genome chromosomes in the F_3 generation. Five randomly selected seeds from each of the 10 individually selected F_2 plants were advanced to the F_3 generation, i.e., 50 F_3 lines for each of the three

crosses. The 10 F_2 families from each cross were chosen based on the D-genome content and the production of sufficient amount of viable seeds. D-genome content ranged from null to at least one copy of each of the seven non-homologous D chromosomes as indicated in Supplementary Table 1. F_4 lines used in this study were obtained by selfing the F_3 lines and advancing one seed of each plant.

Genome-wide DArTseq™ assay of F_4 generation

Fifty lines of each of the Sunguard/Caparoi, Sunguard/Hyperno and Sunguard/WID802 crosses were investigated in the F_4 generation using genome-wide DArTseq™ markers. DNA was extracted from 150 individual F_4 lines using the CTAB method (Saghai-Maroof et al. 1984). The quality assessment and normalisation of DNA for the DArTseq™ assay were carried out as per Padmanaban et al. (2017). A hexaploid wheat microarray with the service tag DW16-2185 was used as a marker source.

Molecular and cytological validation in subsequent F_5 generation

The presence of the 2G segment and D-genome retention in the subsequent F_5 generation was analysed using microsatellite markers as described above. Rearranged D chromosomes identified in selected F_4 lines were validated in the F_5 generation using the genomic in situ hybridisation (GISH) technique. The cytological slide preparation and subsequent GISH were carried out as previously described (Zhang et al. 2004; Padmanaban et al. 2017b).

Statistical analysis

Differences in the inheritance of the 2G segment between crosses were assessed by performing a pairwise comparison test. The relationship between the D-genome retention and 2G inheritance in the F_2 and F_4 generation was tested using a Fisher's test. A chi-square goodness of fit test was conducted to check the quality and segregation distortion of genome sequences developed through DArTseq™. Tukey's post-hoc test was conducted to test the significant differences between the crosses by comparing the mean proportion of parental alleles in the A and B genomes and chromosomal 2G segment inheritance of F_4 progeny. Statistical analysis was conducted using *R* software version 3.3.2 (R Development Core Team 2017) with multiple *R* packages including lme4 (Bates et al. 2014), lmerTest (Kuznetsova et al. 2017), multcomb (Hothorn et al. 2017), PMCMR (Pohlert 2014) and plyr (Wickham 2011).

Table 1 Segregation ratios of the 2G segment in 60 F_2 lines of three crosses (2G+, homozygous for the 2G segment; H, heterozygous; 2G-, homozygous for the 2B segment from durum) are given together with an average and percentage of D-genome chromosomes retained in each cross

	Sunguard/Caparoi	Sunguard/Hyperno	Sunguard/WID802
2G segment			
2G+	14 (32%)	12 (20%)	9 (15%)
H	31 (52%)	26 (43%)	27 (45%)
2G-	15 (26%)	22 (37%)	24 (10%)
Number of D-genome chromosomes			
Null	6 (10%)	8 (13%)	6 (10%)
1	7 (12%)	6 (10%)	15 (25%)
2	8 (13%)	6 (10%)	10 (17%)
3	9 (15%)	9 (15%)	9 (15%)
4	8 (13%)	6 (10%)	7 (12%)
5	12 (20%)	5 (8%)	6 (10%)
6	6 (10%)	10 (17%)	4 (7%)
7	4 (7%)	10 (17%)	3 (5%)

Results

2G inheritance and D-genome retention in the F_2 generation

Inheritance of the 2G segment and retention of D-genome chromosomes were investigated in 60 F_2 lines from each of the crosses. Screening of these 180 F_2 lines with microsatellite markers indicated significant differences in the segregation ratio of the 2G segment in different crosses (Table 1). A segregation ration of 1:2:1 (14:31:15) was observed for the Sunguard/Caparoi cross, whereas a significant distortion towards the durum parent (the absence of the 2G segment) was observed in the Sunguard/Hyperno (12:26:22) and Sunguard/WID802 (9:27:24) crosses.

Significant differences ($P < 0.05$) were observed in the retention of D-genome chromosomes between the crosses (Table 1). D-genome chromosome numbers ranged from the presence of at least one copy of all seven non-homologous D-genome chromosomes (in 7, 17, and 5% of the lines of the Sunguard/Caparoi, Sunguard/Hyperno and Sunguard/WID802 crosses, respectively) to complete absence of D-genome chromosomes (in 10, 13, and 10% of the lines of the Sunguard/Caparoi, Sunguard/Hyperno and Sunguard/WID802 crosses, respectively).

Relationship between 2G inheritance and D-genome retention

A strong correlation ($P < 0.001$) was observed between the 2G segment and the number of D-genome chromosomes retained in the three crosses (Fig. 1). Lines that were homozygous for the 2G segment retained between four and seven non-homologous D-genome chromosomes, while the lines that were homozygous for the tetraploid 2B chromosomal segment in all but one case possessed less than four non-homologous D-genome chromosomes (Fig. 1).

Detection of 2G segment in F_4 generation

To determine the presence of the 2G introgression in F_4 lines, 1094 unique polymorphic DArT sequences on chromosome 2B were utilised. The introgressed 2G segment was located between 59 and 103 cM, which is approximately 42 cM in length according to the map supplied by DArT Pty Ltd.

The 2G segment, which was present in 21 of the 30 lines that were investigated in the F_2 generation (of which 13 were heterozygous and eight homozygous for the 2G introgression), was present in only eight of the 30 families (27%) in the F_4 generation (Table 2). The 13 lines that were heterozygous for the 2G segment in the F_2 generation had lost the 2G segment and were homozyogus for the durum 2B segment in the F_4 generation. No recombinations were detected within the 2G segment suggesting that the introgression was transferred as a whole segment.

Most of the lines of the F_4 generation either had a complete durum set of chromosomes ($2n = 4\times = 28$) or had at least one copy of each of the seven non-homologous D chromosomes (Table 2). Only two out of 50 lines from the Sunguard/Hyperno cross retained at least one copy of each of the seven D chromosomes. Fifty and 40% of the Sunguard/Caparoi and Sunguard/

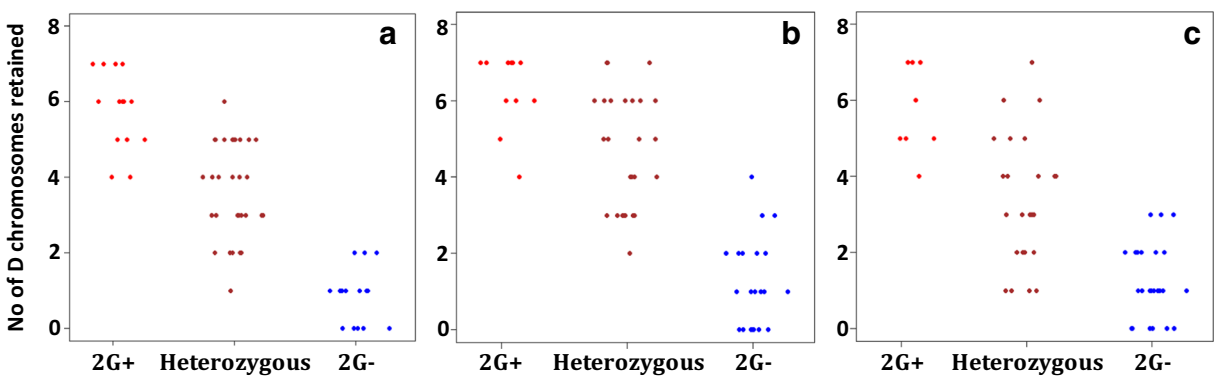
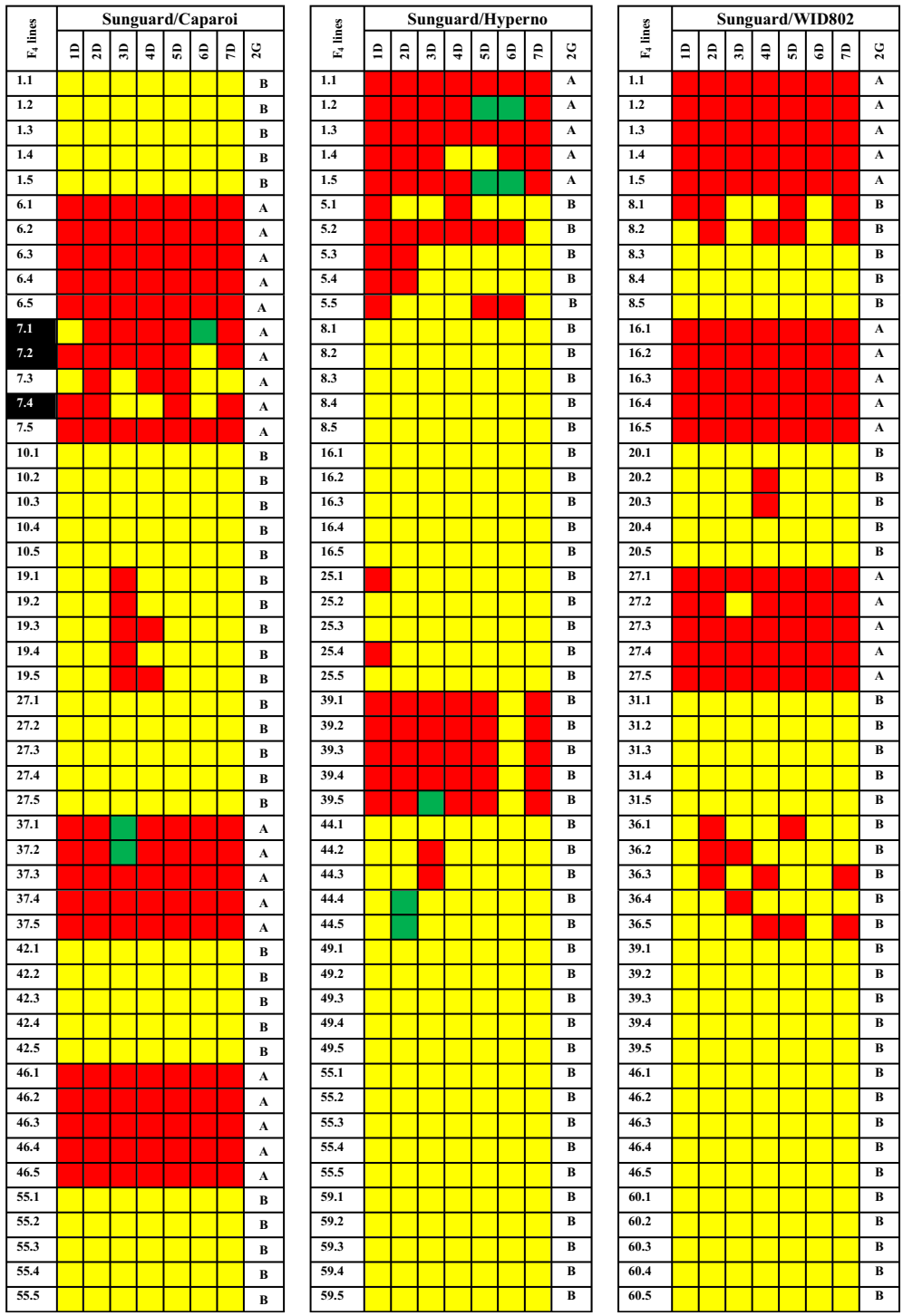


Fig. 1 Relationship between the inheritance of the 2G segment and the retention of D-genome chromosomes in 60 F_2 progeny from each of the three crosses (**a** Sunguard/Caparoi, **b** Sunguard/Hyperno, **c** Sunguard/WID802). 2G+ lines are homozygous for the 2G segment and 2G- lines are homozygous for the 2B segment from durum

Table 2 Number of D-genome chromosomes retained in 10 F_4 families of each of the Sunguard crosses. Each row represents an individual line and each column represents a non-homologous D chromosome. Red and yellow colors represent the presence and absence of D chromosomes, respectively. Inheritance of the 2G segment for each Sunguard cross is given with A and B indicating the presence and absence of the 2G segment, respectively. Lines highlighted in the Sunguard/Caparoi cross were advanced to the F_5 generation. Incomplete chromosomes are highlighted in green



WID802 F_4 families, respectively, had lost the D-genome chromosomes completely (Table 2).

Rearranged D chromosomes were observed in eight of 150 F_4 progenies, three from the Sunguard/Caparoi and five from the Sunguard/Hyperno cross (Table 2). Cytological tests were conducted on six of these F_5 lines (7.1, 37.1 and 37.2 from the Sunguard/Caparoi cross and 39.5, 44.1 and 44.5 from the Sunguard/Hyperno cross). GISH results confirmed the presence of telocentric chromosomes in lines 7.1, 37.1 and 37.2 (Fig. 2). A pair of whole arm translocations between A and B chromosomes and a single telocentric 3DL with intact centromere were observed in line 39.5. Furthermore, translocations were observed between A and D chromosomes in lines 44.4 and 44.5. Both of these lines possessed a stable durum set of chromosomes (2n = × 4 = 28) with a 2DS segment translocated onto an A-genome chromosome (Fig. 2).

Association of introgressed 2G segment with D-genome chromosomes

A Fisher's test was conducted to confirm that there was still a strong correlation ($P < 0.001$) between the introgressed 2G segment and the retention of D-genome chromosomes in the F_4 generation (Figs. 3). Only six of the 40 lines that inherited the 2G segment did not have a full set of D-genome chromosomes (Table 2). Three of these lines produced viable seeds and were advanced to the F_5 generation (Supplementary Table 2). Marker analysis of the F_5 generation confirmed that the 2G segment was present in a homozygous status in all of these lines. The dominant marker gwm501 positioned at the distal end of the 2G segment was absent in two of the lines (Supplementary Table 2). Three lines were identified which had retained only two (chromosomes 3D and 7D and 4D and 6D, respectively) or three D-genome chromosomes (1D, 5D and 6D).

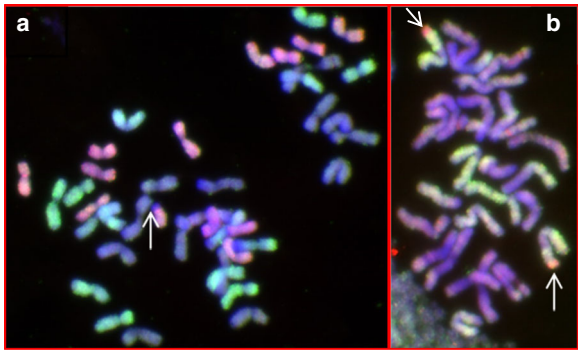


Fig. 2 Multi-coloured GISH analyses of rearranged D chromosomes in selected F_5 lines of the Sunguard/Caparoi and Sunguard/Hyperno crosses. **a** Line number 6.5 with arrow indicating a telocentric 3DL chromosome. **b** Stable durum line number 7.4 with 2DS segment translocated to a pair of A-genome chromosomes (indicated by arrows). A-genome chromosomes are green, B-genome chromosomes are blue and D-genome chromosomes are pink in colour

Discussion

Three sets of crosses were developed to investigate the presence of the *T. timopheevii* 2G segment in the progeny of inter-ploidy crosses. Sunguard, a current commercial bread wheat variety, which harbours the 2G segment was used as the maternal parent to cross with three different durum varieties. Differences were observed between the crosses in the segregation ratios of the 2G segment with only the Sunguard/Caparoi cross segregating in the expected 3:1 ratio for the 2G segment. In the Sunguard/Hyperno and Sunguard/WID802 crosses, segregation distortion towards the durum chromosome 2B was observed.

The DArT analysis of the subsequent F_4 generation confirmed preferential retention of the durum chromosome 2B with all heterozygous F_2 lines having become homozygous for the durum 2B chromosome in the F_4 generation. These results differ from

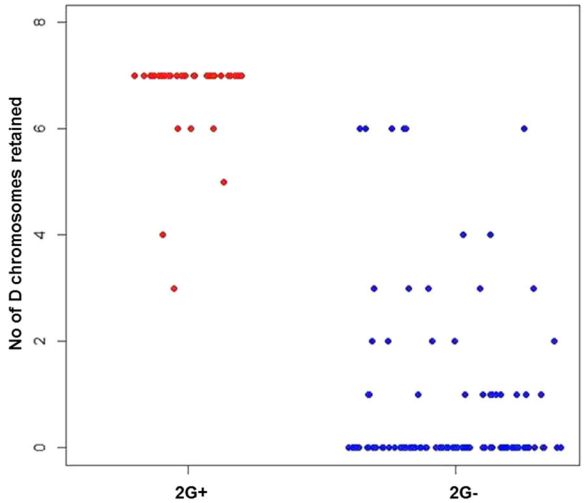


Fig. 3 Relationship between the inheritances of 2G segment and the retention of D-genome chromosomes in 150 F_4 lines; 2G+ lines are homozygous for the 2G segment and 2G-lines are homozygous for the 2B segment from durum

previous reports on hexaploid/hexaploid wheat crosses, where one parent possesses the 2G segment. In these crosses, a significant segregation distortion towards the retention of the 2G segment was observed (Kammholz et al. 2001; Kammholz et al. 1998; Bovill et al. 2006; Bovill et al. 2010).

Lines homozygous for the 2G segment had retained a large number of D-genome chromosomes ranging from four to seven. The F_2 lines that were heterozygous for the 2G segment had an intermediate D-genome number ranging from three to six. This suggests that hexaploid/tetraploid derived lines, which have eliminated the durum 2B segment and retained the 2G segment, may need to be compensated by retaining a large number of D-genome chromosomes. DArT analysis in the advanced F_4 population confirmed that there is a strong relationship between the introgressed 2G segment and D-genome retention, which suggests that it may be difficult to introduce the 2G segment into a tetraploid durum background. Whether this D-genome association is only occurring with the 2G introgression or also with other bread wheat introgressions needs to be further investigated.

The 2G segment was transmitted as a whole without any chromosomal cross-overs. This is similar to another study, where the translocation was transferred between bread wheats as a whole segment (Lehmensiek et al. 2005). Using Sunguard as the donor parent to introduce the 2G segment into durum may not be ideal as only a small number of progeny segregated for the 2G segment and most of these lines had a full set of D-genome chromosomes. Other 2G donors should be tested to determine whether this is the same with all 2G donors. However, in a previous study using bread wheat Sunco as the 2G donor parent, the 2G segment was not transferred to the progeny in two of the four Sunco/durum crosses (Martin et al. 2013). The pedigrees of Sunguard (Sun289E/Sr2Janz) and Sunco (SUN9E27*4/3AG14//WW15/3/3*COOK) are different, suggesting that the transfer of the 2G segment into durum is not strongly influenced by the maternal parent.

Six F_4 lines from the Sunguard/Caparoi and Sunguard/Hyperno crosses had inherited the 2G segment and did not have a full set of D-genome chromosomes. Because these lines did not retain complete D-genome sets, there is a high chance of D chromosomes being eliminated in future generations. Unfortunately, viable seed could only be obtained from three of these lines. They will be screened for crown rot resistance in the near future and may be of interest to durum breeding programs as they may also contain other useful traits.

Similar to previous studies, telocentric chromosomes with intact centromeres were observed in a number of F_5 lines (Koo et al. 2015; Padmanaban et al. 2018). The present study also validated two durum lines from the Sunguard/Hyperno cross with 2DS translocated to an A-genome chromosome. These lines with an extra 2DS segment may have traits which could be potentially useful for durum breeding in future.

To obtain tetraploid durum lines (2n=4X=28) containing the 2G segment further selfing of F_5 lines that were homozygous for the 2G segment and only had four or less non-homologous D-genome chromosomes may need to be undertaken to eliminate remaining D chromosomes. However, a previous study has indicated that paired D-chromosomes may become stable in later generations (Padmanaban et al. 2018). Backcrossing with the durum parent may be another approach to eliminate remaining D chromosomes; however, this could result in the loss of the 2G translocation. An alternative option is to cross F_5 lines that are homozygous for the 2G segment and have different copies of D-genome chromosomes. The progenies derived from these crosses are likely to inherit any single non-homologous D chromosome, which may be eliminated in subsequent generations of selfing. Irrespective of which strategy is chosen to obtain tetraploid lines large-sized populations need to be developed to increase the chances of obtaining lines, which have the introgression and a low number of or no D chromosomes. Overall, this study has shown that the 2G segment and possibly other alien translocations can be transferred to the durum wheat background through hexaploid/tetraploid wheat crosses; however, different strategies may need to be considered for the elimination of D-genome chromosomes.

Funding information This study was partly funded by the Grain Research and Development Corporation, Australia. An Australian Postgraduate Award supported Sriram Padmanaban's Ph.D. study.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

Appels R (2003) A consensus molecular genetic map for wheat—a cooperative international effort. In: Proceedings of the 10th International Wheat Genetics Symposium, pp 211–214

Bariana HS, Hayden MJ, Ahmed NU, Bell JA, Sharp PJ, McIntosh RA (2001) Mapping of durable adult plant and seedling resistances to stripe rust and stem rust diseases in wheat. Aust J Agric Res 52:1247–1255

Bates D, Maechler M, Bolker B, Walker S (2014) lme4: linear mixed-effects models using Eigen and S4. R package version 1:1–23

Bovill W, Ma W, Ritter K, Collard B, Davis M, Wildermuth G, Sutherland M (2006) Identification of novel QTL for resistance to crown rot in the doubled haploid wheat population 'W21MMT70'×'Mendos. Plant Breed 125:538–543

Bovill WD, Horne M, Herde D, Davis M, Wildermuth GB, Sutherland M (2010) Pyramiding QTL increases seedling resistance to crown rot (*Fusarium pseudograminearum*) of wheat (*Triticum aestivum*). Theor Appl Genet 121:127–136

Dundas I, Anugrahwati D, Verlin D, Park R, Bariana H, Mago R, Islam A (2007) New sources of rust resistance from alien species: meliorating linked defects and discovery. Crop & Pasture Sci 58:545–549

Eberhard FS, Zhang P, Lehmensiek A, Hare RA, Simpfendorfer S, Sutherland MW (2010) Chromosome composition of an F_2 *Triticum aestivum* × *T. turgidum* spp. *durum* cross analysed by DArT markers and MCFISH. Crop & Pasture Sci 61:619–624

Friebe B, Jiang J, Raupp W, McIntosh R, Gill B (1996) Characterization of wheat-alien translocations conferring resistance to diseases and pests: current status. Euphytica 91:59–87

Hothorn T, Bretz F, Westfall P, Heiberger RM, Schuetzenmeister A, Scheibe S, Hothorn MT (2017) Package 'multcomp'. Obtenido de http://cran.statsfu.ca/web/packages/multcomp/multcomp

Jiang J, Friebe B, Gill BS (1993) Recent advances in alien gene transfer in wheat. Euphytica 73:199–212

Kammholz SJ, Grams RA, Banks PM, Sutherland MW (1998) Segregation of glutenins in wheat × maize-derived doubled haploid wheat populations. Aust J Agric Res 49:1253–1260

Kammholz SJ, Campbell A, Sutherland M, Hollamby G, Martin P, Eastwood R, Barclay I, Wilson R, Brennan P, Sheppard J (2001) Establishment and characterisation of wheat genetic mapping populations. Aust J Agric Res 52:1079–1088

Koo D-H, Sehgal SK, Friebe B, Gill BS (2015) Structure and stability of telocentric chromosomes in wheat. PLoS One 10: e0137747

Kuznetsova A, Brockhoff PB, Christensen RHB (2017) lmerTest package: tests in linear mixed effects models. J Stat Softw 82:13

Lehmensiek A, Campbell A, Sutherland M, Williamson P, Michalowitz M, Daggard G (2004) QTLs for black-point resistance in wheat and the identification of potential markers for use in breeding programmes. Plant Breed 123:410–416

Lehmensiek A, Eckermann PJ, Verbyla AP, Appels R, Sutherland MW, Daggard GE (2005) Curation of wheat maps to improve map accuracy and QTL detection. Aust J Agric Res 56:1347–1354

Lehmensiek A, Eckermann PJ, Verbyla AP, Appels R, Sutherland MW, Martin D, Daggard GE (2006) Flour yield QTLs in three Australian doubled haploid wheat populations. Aust J Agric Res 57:1115–1122

Leonova I, Röder M, Budashkina E, Kalinina N, Salina E (2002) Molecular analysis of leaf rust-resistant introgression lines obtained by crossing of hexaploid wheat *Triticum aestivum* with tetraploid wheat *Triticum timopheevii*. Russ J Genet 38: 1397–1403

Leonova I, Laikova L, Popova O, Unger O, Börner A, Röder M (2007) Detection of quantitative trait loci for leaf rust resistance in wheat—*T. timopheevii*/*T. tauschii* introgression lines. Euphytica 155:79–86

Mago R, Miah H, Lawrence G, Wellings C, Spielmeyer W, Bariana H, McIntosh R, Pryor A, Ellis J (2005) High-resolution mapping and mutation analysis separate the rust resistance genes *Sr31*, *Lr26* and *Yr9* on the short arm of rye chromosome 1. Theor Appl Genet 112:41–50

Martin A, Simpfendorfer S, Hare RA, Eberhard FS, Sutherland MW (2011) Retention of D genome chromosomes in pentaploid wheat crosses. Heredity 107:315–319

Martin A, Simpfendorfer S, Hare R, Sutherland M (2013) Introgression of hexaploid sources of crown rot resistance into durum wheat. Euphytica 192:463–470

Molnár-Láng M, Ceoloni C, Doležel JE (2015) Alien introgression in wheat. Cytogenetics, molecular biology, and genomics. Springer, Cham

Olson EL, Brown-Guedira G, Marshall DS, Jin Y, Mergoum M, Lowe I, Dubcovsky J (2010) Genotyping of U.S. wheat germplasm for presence of stem rust resistance genes *Sr24*, *Sr36* and $Sr1RS^{Amigo}$. Crop Sci 50:668–675

Padmanaban S, Sutherland MW, Knight NL, Martin A (2017a) Genome inheritance in populations derived from hexaploid/tetraploid and tetraploid/hexaploid wheat crosses. Mol Breed 37:48

Padmanaban S, Zhang P, Hare RA, Sutherland MW, Martin A (2017b) Pentaploid wheat hybrids: applications, characterisation, and challenges. Front Plant Sci 8:358

Padmanaban S, Zhang P, Sutherland MW, Knight NL, Martin A (2018) A cytological and molecular analysis of D-genome chromosome retention in the F_2 to F_6 generation of hexaploid/tetraploid wheat crosses. Crop & Pasture Sci 69:121–130

Pohlert T (2014) The pairwise multiple comparison of mean ranks package (PMCMR). R package:2004–2006

R Development Core Team (2017) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna

Riley R, Chapman V (1967) The inheritance in wheat of cross-ability with rye. Genet Res 9:259–267

Saghai-Maroof MA, Soliman KM, Jorgensen RA, Allard R (1984) Ribosomal DNA spacer-length polymorphisms in barley: Mendelian inheritance, chromosomal location, and population dynamics. Proc Natl Acad Sci U S A 81:8014–8018

Tao W, Liu D, Liu J, Feng Y, Chen P (2000) Genetic mapping of the powdery mildew resistance gene *Pm6* in wheat by RFLP analysis. Theor Appl Genet 100:564–568

Wickham H (2011) The split-apply-combine strategy for data analysis. J Stat Softw 40:1–29

Wulff BB, Moscou MJ (2014) Strategies for transferring resistance into wheat: from wide crosses to GM cassettes. Front Plant Sci 5:692

Zhang P, Li W, Friebe B, Gill BS (2004) Simultaneous painting of three genomes in hexaploid wheat by BAC-FISH. Genome 47:979–987

Supplementary Material: Supplementary Table-1 Retention of D-genome chromosomes in 180 F2 progeny of three crosses. Each row represents an individual line and each column represents a unique D chromosome. Red and yellow colours indicate the presence and absence of D chromosomes, respectively. Highlighted lines from each cross were advanced to F4 generation. Inheritance of the 2G segment in each of the crosses is shown (A = maternal, H= heterozygous and B = paternal genotype)

F_2 lines	Sunguard/Caparoi									F_2 lines	Sunguard/Hyperno									F_2 lines	Sunguard/WID802								
	1D	2D	3D	4D	5D	6D	7D	All	2G		1D	2D	3D	4D	5D	6D	7D	All	2G		1D	2D	3D	4D	5D	6D	7D	All	2G
1								1	B	1								7	A	1								7	A
2								2	B	2								7	A	2								3	H
3								2	H	3								4	H	3								6	A
4								4	H	4								7	H	4								3	H
5								5	H	5								6	H	5								3	H
6								7	A	6								7	H	6								1	B
7								7	A	7								0	B	7								1	B
8								5	H	8								3	H	8								5	H
9								3	H	9								6	H	9								1	H
10								0	B	10								7	A	10								2	B
11								3	H	11								5	H	11								4	H
12								2	B	12								6	H	12								0	B
13								1	B	13								3	H	13								5	A
14								6	A	14								2	B	14								2	H
15								5	A	15								1	B	15								1	B
16								5	H	16								2	B	16								7	A
17								4	H	17								3	H	17								4	H
18								6	A	18								1	B	18								3	H
19								3	H	19								3	H	19								0	B
20								4	H	20								7	H	20								2	B
21								1	B	21								0	B	21								5	A
22								6	H	22								1	B	22								6	A
23								1	B	23								2	B	23								2	B
24								3	H	24								7	A	24								1	B
25								3	H	25								4	H	25								1	B
26								5	H	26								6	H	26								2	B
27								4	H	27								7	A	27								7	A
28								4	H	28								0	B	28								1	B
29								2	H	29								1	B	29								3	B
30								3	H	30								1	B	30								3	H
31								1	B	31								4	H	31								4	H
32								3	H	32								3	H	32								5	H
33								0	B	33								6	H	33								0	B
34								5	H	34								0	B	34								0	B
35								4	A	35								6	A	35								1	H
36								2	B	36								6	A	36								6	H
37								7	A	37								3	B	37								0	B
38								4	A	38								7	A	38								1	H
39								6	A	39								6	H	39								0	B
40								0	B	40								0	B	40								5	H
41								1	B	41								5	A	41								2	H
42								3	H	42								5	H	42								4	H
43								3	H	43								0	B	43								1	H
44								5	A	44								5	H	44								4	H
45								5	H	45								0	B	45								2	B
46								7	A	46								3	H	46								1	B
47								6	A	47								4	B	47								1	B
48								1	H	48								3	H	48								1	H
49								5	H	49								3	B	49								2	H
50								4	H	50								7	H	50								4	H
51								6	A	51								4	H	51								4	A
52								5	H	52								6	A	52								3	H
53								0	B	53								6	A	53								3	B
54								5	A	54								2	B	54								1	B
55								2	H	55								2	H	55								2	H
56								2	H	56								1	B	56								2	B
57								0	B	57								2	B	57								5	A
58								5	H	58								5	H	58								6	H
59								2	H	59								0	B	59								3	H
60								0	B	60								4	A	60								1	B

Chapter 6

Investigating mitochondrial inheritance in pentaploid crosses

Introduction

The majority of the genetic information in the plant system resides in the nucleus (nuclear genome), although a minor portion is also stored in the cytoplasm (cytoplasmic genomes). The cytoplasmic organelles, including mitochondria and chloroplast, have their own genome and their functions are highly dependent on the nuclear genome (Hattori et al. 2002). The coordination between the nucleus and the cytoplasm is vital for gene expression to ensure all the mitochondria and chloroplastic proteins evolve correctly and without delay to perform normally (Bonen and Bird 1988; Cannino et al. 2007). Mitochondria are an important constituent of all eukaryotic cells and provide a critical function that is involved in the synthesis of essential phospholipids and in signaling various cellular pathways. Mitochondria are also responsible for generating energy in the form of adenosine triphosphate (ATP), which is readily used by the plant system as a source of chemical energy for enabling various elements of growth and development (Bonen and Bird 1988).

The inheritance of genetic material from the parents differs between the cytoplasmic and nuclear genome. Unlike nucleoplasm, where inheritance of the parental genome is strictly bi-parental, the cytoplasm (plasmon) inherits purely uniparental maternal material (Birky 1995). In most of the plant species, the maternal inheritance of cytoplasmic DNA has been well studied (Birky 1995; Birky 2001; Whatley 1982; Reboud and Zeyl 1994). A notable cellular event which favors the maternal inheritance of mitochondrial DNA, such as degradation of mt DNA in male gametic cells is one of the major pathways for uniparental inheritance. This degradation of mt DNA can be observed under fluorescent microscopy (Miyamura et al. 1987). Interestingly this degradation has not been observed in species in which bi-parental mt DNA inheritance is observed (Nagata et al. 1999).

The generative pollen cells receive mitochondria after the first mitosis of the pollen cells. Mitochondria remain structurally uninjured in the mature pollen cells. Physical exclusion of male gametic cytoplasm before and during fertilisation significantly reduces the levels of mitochondrial DNA in pollen cells that fuse with the embryo and

this is another argument for maternal inheritance (Sato and Sato 2013). However, paternal sequences or bi-parental inheritance of mitochondrial gene sequences have been witnessed in a number of inter or intraspecific hybrids (Nagata 2010). The nucleus cytoplasmic interaction (NCI) is the condition where the nucleus of the one species is combined with the cytoplasm of others to produce alloplasmic hybrids (Nagata 2010; Laser et al. 1997; Kmiec et al. 2006; Hattori et al. 2002). These alloplasmic lines that developed through the combination of nucleus-cytoplasm of different species often express male sterility (sterile anthers) which affects self-pollination. The male sterility observed in the alloplasmic NC hybrids is often associated with the expression of novel mitochondrial genes with one or more nucleotide rearrangement that alter the protein coding sequences (Hattori et al 2002). Thus the novel expression of mitochondrial genes is likely to be responsible for male sterility in inter or intraspecific hybrids and this is mainly linked to the mode of organelle genome inheritance.

Several investigations have focussed on the mitochondrial inheritance of inter and intra ploidy wheat crosses, for example, intra-ploidy wheat crosses between rye (2x) (Laser et al. 1997), and barley (Aksyonova et al. 2005) and inter-ploidy wheat crosses between *Aegilops* sp. (2x,4x,6x) (Hattori et al. 2002), and *T. timopheevi* (4x) (Kitagawa et al. 2003). Paternal identical and paternally derived sequences were observed in NC hybrids, and the apparent absence of such sequences in the maternal parents confirmed the paternal contribution of mt DNA to developed NC hybrids (Hattori et al. 2002; Laser et al. 1997). To date, the mitochondrial inheritance of pentaploid derived hexaploid/tetraploid or tetraploid/hexaploid wheat crosses has not been investigated. Thus the present study has focussed on the cytoplasmic inheritance in pentaploid derived wheat hybrids to understand the maternal inheritance of mt DNA. In order to determine the inheritance of hexaploid or tetraploid mitochondrial fragments, it is necessary to identify polymorphic differences in the mitochondrial DNA between these two ploidy level species. This can be achieved by making use of bread (Cui et al. 2009) and durum (Noyszewski et al. 2014) wheat whole mitochondrial genome assemblies (Bonen and Bird 1988) publically available through the NCBI website.

The total length of the bread and durum wheat mitochondrial genomes is 452 528 and 451678bp, respectively. Recently developed durum wheat whole mitochondrial genome assembly is closely identical to the previously sequenced bread wheat mitochondrial

genome (Noyszewski et al. 2014). Although the genome sequences are closely matching, 40 single nucleotide differences (SNP's) have been detected between bread and durum wheat, of which five were in known mitochondrial genes: *rps*1, *rps2, cox3* and *ccmFn* (Noyszewski et al. 2014; Calixte and Bonen 2008; Chapdelaine and Bonen 1991). The comparative analysis has indicated seven SNP's (4 transversions and 3 transitions) and 10 indels (insertions and deletions) when aligned with the other bread wheat cultivar, Chinese Spring (Cui et al. 2009). Furthermore, a comparative analysis of the mitochondrial genome of a cytoplasmic male sterile line and its maintainer line, bread wheat cultivar, Chinese Yumai, is also available (Liu et al. 2011).

Using the available information, this study aimed to prove the hypothesis that mt DNA inherits maternally. The objectives of the present study were: (i) to identify allelic difference (SNP'S) in the targeted mt region of bread and durum mt DNA using publically available sequences; (ii) to amplify and sequence the identified heteroplasmic mt DNA region in the current Australian bread and durum wheat cultivars and in a unique set of hexaploid/tetraploid and tetraploid/hexaploid wheat crosses to confirm maternal inheritance.

Materials and Methods

The plant materials used include the Australian hexaploid bread wheat lines 2-49, Sunco, Sunguard, LRC2010-70, LRC2010-150, W21MMT70, and doubled haploid lines B34 (Sunco/2-49), and E25 (2-49/W21MMT70) Australian tetraploid durum wheat lines Bellaroi, Parent B (950329), Hyperno, Caparoi, and WID802. The F_2 lines of hexaploid/tetraploid and tetraploid/hexaploid crosses with LRC2020-150 and WID802 as parents were used to test the maternal inheritance of mt DNA. These crosses were previously studied for genome inheritance (Padmanaban et al., 2017b). The pedigree details of the bread and durum wheat cultivars used in the present study can be found in Figure-1.

Figure-1 Sanger sequencing alignment for *nad*-3, gene of eight bread and four durum cultivars. The table also contains the consensus raw sequences obtained from the NCBI for designing primer and the pedigree details of the bread and durum wheat cultivars.

Parent 1 **Sunguard (Sun289E/Sr2Janz)**

Parent 2 **Sunco (SUN9E27*4/3AG14//WW15/3/3*COOK)**

Parent 3 **LRC2010-70 (QT10162/W21MMT70//QT10162)**

Parent 4 LRC2010-150(GW95-703*C15/Lang)

Parent 5 2-49 (Gala/Gluyas Early)

Parent 6 W21MMT70 (A Western Australian line of unknown pedigree)

Parent 7 B34 Double Haploid (Sunco/2-49)

Parent 8 E25 Double haploid (2-49/W21MMT70)

Parent 9 Parent B (920196/920357)

Parent 10 Caparoi (LY2.6.3/930054)

Parent 11 Hyperno (Kalka sister line/Tamaroi)

Parent 12 WID-802
(Syrica1/Yallaroi//Tamaroi/Lingzhi/Yallaroi*2///RAC875/Kalka//Tamaroi////Lingzhi/Yallaroi//Tamaroi///Lingzhi/Yallaroi)

(Note hexaploid bread wheat parents were highlighted in red colour and tetraploid durum parent were highlighted in yellow colour)

```
Parent1_Nad1_A01        ----AAGGGGGGAAGGACATAGGAAAGAGGGATGCCTACAAAAAATCAATTGATTCGTCA
Parent2_Nad1_A02        GGGCAAGGGGGGAAGGACATAGGAAAGAGGGATGCCTACAAAAAATCAATTGATTCGTCA
Parent3_Nad1_A03        -GGCAAGGGGGGAAGGACATAGGAAAGAGGGATGCCTACAAAAAATCAATTGATTCGTCA
Parent4_Nad1_A04        GGGCAAGGGGGGAAGGACATAGGAAAGAGGGATGCCTACAAAAAATCAATTGATTCGTCA
Parent5_Nad1_A05        -GGCAAGGGGGGAAGGACATAGGAAAGAGGGATGCCTACAAAAAATCAATTGATTCGTCA
Parent6_Nad1_A06        -------GGGGGAAGGACATAGGAAAGAGGGATGCCTACAAAAAATCAATTGATTCGTCA
Parent7_Nad1_A07        ------------------------------------------------------------
Parent8_Nad1_A08        ----AAGGGGGGAAGGACATAGGAAAGAGGGATGCCTACAAAAAATCAATTGATTCGTCA
Parent9_Nad1_A09        -GGCAAGGGGGGAAGGACATAGGAAAGAGGGATGCCTACAAAAAATCAATTGATTCGTCA
Parent10_Nad1_A10       GGGCAAGGGGGGAAGGACATAGGAAAGAGGGATGCCTACAAAAAATCAATTGATTCGTCA
Parent11_Nad1_A11       -GGCAAGGGGGGAAGGACATAGGAAAGAGGGATGCCTACAAAAAATCAATTGATTCGTCA
Parent12_Nad1_A12       -GGCAAGGGGGGAAGGACATAGGAAAGAGGGATGCCTACAAAAAATCAATTGATTCGTCA

Parent1_Nad1_A01        TGGTAGAGAAGAAAAACGGCGCACGGACCGTACTCGAGCTTCGGATCAATGTCCCCAGAA
Parent2_Nad1_A02        TGGTAGAGAAGAAAAACGGCGCACGGACCGTACTCGAGCTTCGGATCAATGTCCCCAGAA
Parent3_Nad1_A03        TGGTAGAGAAGAAAAACGGCGCACGGACCGTACTCGAGCTTCGGATCAATGTCCCCGGAA
Parent4_Nad1_A04        TGGTAGAGAAGAAAAACGGCGCACGGACCGTACTCGAGCTTCGGATCAATGTCCCCAGAA
Parent5_Nad1_A05        TGGTAGAGAAGAAAAACGGCGCACGGACCGTACTCGAGCTTCGGATCAATGTCCCCAGAA
Parent6_Nad1_A06        TGGTAGAGAAGAAAAACGGCGCACGGACCGTACTCGAGCTTCGGATCAATGTCCCCGGAA
Parent7_Nad1_A07        ------------------------------------------------------------
Parent8_Nad1_A08        TGGTAGAGAAGAAAAACGGCGCACGGACCGTACTCGAGCTTCGGATCAATGTCCCCAGAA
Parent9_Nad1_A09        TGGTAGAGAAGAAAAACGGCGCACGGACCGTACTCGAGCTTCGGATCAATGTCCCCAGAA
Parent10_Nad1_A10       TGGTAGAGAAGAAAAACGGCGCACGGACCGTACTCGAGCTTCGGATCAATGTCCCCAGAA
Parent11_Nad1_A11       TGGTAGAGAAGAAAAACGGCGCACGGACCGTACTCGAGCTTCGGATCAATGTCCCCAGAA
Parent12_Nad1_A12       TGGTAGAGAAGAAAAACGGCGCACGGACCGTACTCGAGCTTCGGATCAATGTCCCCAGAA

Parent1_Nad1_A01        GCAAGGAGTATGCCTGCGTGTTTCGACGAGAACACCGAAAAAACCTAATTCAGCTCTACG
Parent2_Nad1_A02        GCAAGGAGTATGCCTGCGTGTTTCGACGAGAACACCGAAAAAACCTAATTCAGCTCTACG
Parent3_Nad1_A03        GCAAGGAGTATGCCTGCGTGTTTCGACGAGAACACCGAAAAAACCTAATTCAGCTCTACG
Parent4_Nad1_A04        GCAAGGAGTATGCCTGCGTGTTTCGACGAGAACACCGAAAAAACCTAATTCAGCTCTACG
Parent5_Nad1_A05        GCAAGGAGTATGCCTGCGTGTTTCGACGAGAACACCGAAAAAACCTAATTCAGCTCTACG
Parent6_Nad1_A06        GCAAGGAGTATGCCTGCGTGTTTCGACGAGAACACCGAAAAAACCTAATTCAGCTCTACG
Parent7_Nad1_A07        ------------------------------------------------------------
Parent8_Nad1_A08        GCAAGGAGTATGCCTGCGTGTTTCGACGAGAACACCGAAAAAACCTAATTCAGCTCTACG
Parent9_Nad1_A09        GCAAGGAGTATGCCTGCGTGTTTCGACGAGAACACCGAAAAAACCTAATTCAGCTCTACG
Parent10_Nad1_A10       GCAAGGAGTATGCCTGCGTGTTTCGACGAGAACACCGAAAAAACCTAATTCAGCTCTACG
Parent11_Nad1_A11       GCAAGGAGTATGCCTGCGTGTTTCGACGAGAACACCGAAAAAACCTAATTCAGCTCTACG
Parent12_Nad1_A12       GCAAGGAGTATGCCTGCGTGTTTCGACGAGAACACCGAAAAAACCTAATTCAGCTCTACG

Parent1_Nad1_A01        TAAGATAGCAAAAGTACGGTTGAGCAATCGACATGATATATTTGCTCACATTCCAGGCGA
Parent2_Nad1_A02        TAAGATAGCAAAAGTACGGTTGAGCAATCGACATGATATATTTGCTCACATTCCAGGCGA
Parent3_Nad1_A03        TAAGATAGCAAAAGTACGGTTGAGCAATCGACATGATATATTTGCTCACATTCCAGGCGA
Parent4_Nad1_A04        TAAGATAGCAAAAGTACGGTTGAGCAATCGACATGATATATTTGCTCACATTCCAGGCGA
Parent5_Nad1_A05        TAAGATAGCAAAAGTACGGTTGAGCAATCGACATGATATATTTGCTCACATTCCAGGCGA
```

```
Parent6_Nad1_A06        TAAGATAGCAAAAGTACGGTTGAGCAATCGACATGATATATTTGCTCACATTCCAGGCGA
Parent7_Nad1_A07        -----------AAGTACGGTTGAGCAATCGACATGATATATTTGCTCACATTCCAGGAGA
Parent8_Nad1_A08        TAAGATAGCAAAAGTACGGTTGAGCAATCGACATGATATATTTGCTCACATTCCAGGCGA
Parent9_Nad1_A09        TAAGATAGCAAAAGTACGGTTGAGCAATCGACATGATATATTTGCTCACATTCCAGGCGA
Parent10_Nad1_A10       TAAGATAGCAAAAGTACGGTTGAGCAATCGACATGATATATTTGCTCACATTCCAGGCGA
Parent11_Nad1_A11       TAAGATAGCAAAAGTACGGTTGAGCAATCGACATGATATATTTGCTCACATTCCAGGCGA
Parent12_Nad1_A12       TAAGATAGCAAAAGTACGGTTGAGCAATCGACATGATATATTTGCTCACATTCCAGGCGA
                                  ******************************************** **

Parent1_Nad1_A01        AGGTCATAATTCGCAGGAACATTCTATAGTCTTAGTCAGAGGAGGTAGAGTGAAAGATTC
Parent2_Nad1_A02        AGGTCATAATTCGCAGGAACATTCTATAGTCTTAGTCAGAGGAGGTAGAGTGAAAGATTC
Parent3_Nad1_A03        AAGTCATAATTCGCAGGAACATTCTATAGTCTTAGTCAGAGGAGGTAGAGTGAAAGATTC
Parent4_Nad1_A04        AGGTCATAATTCGCAGGAACATTCTATAGTCTTAGTCAGAGGAGGTAGAGTGAAAGATTC
Parent5_Nad1_A05        AGGTCATAATTCGCAGGAACATTCTATAGTCTTAGTCAGAGGAGGTAGAGTGAAAGATTC
Parent6_Nad1_A06        AAGTCATAATTCGCAGGAACATTCTATAGTCTTAGTCAGAGGAGGTAGAGTGAAAGATTC
Parent7_Nad1_A07        AGGTCATAATTCGCAGGAACATTCTATAGTCTTAGTCAGAGGAGGTAGAGTGAAAGATTC
Parent8_Nad1_A08        AGGTCATAATTCGCAGGAACATTCTATAGTCTTAGTCAGAGGAGGTAGAGTGAAAGATTC
Parent9_Nad1_A09        AGGTCATAATTCGCAGGAACATTCTATAGTCTTAGTCAGAGGAGGTAGAGTGAAAGATTC
Parent10_Nad1_A10       AGGTCATAATTCGCAGGAACATTCTATAGTCTTAGTCAGAGGAGGTAGAGTGAAAGATTC
Parent11_Nad1_A11       AGGTCATAATTCGCAGGAACATTCTATAGTCTTAGTCAGAGGAGGTAGAGTGAAAGATTC
Parent12_Nad1_A12       AGGTCATAATTCGCAGGAACATTCTATAGTCTTAGTCAGAGGAGGTAGAGTGAAAGATTC
                        ************************************************************

Parent1_Nad1_A01        GCCAGGTGTGAAATCCCATCGTATTCGAGGAGTCAAGGATTTGCTGGGATTCCCCGGATC
Parent2_Nad1_A02        GCCAGGTGTGAAATCCCATCGTATTCGAGGAGTCAAGGATTTGCTGGGATTCCCCGGATC
Parent3_Nad1_A03        GCCAGGTGTGAAATCCCATCGTATTCGAGGAGTCAAGGATTTGCTGGGATTCCCCGGATC
Parent4_Nad1_A04        GCCAGGTGTGAAATCCCATCGTATTCGAGGAGTCAAGGATTTGCTGGGAATCCCCGGATC
Parent5_Nad1_A05        GCCAGGTGTGAAATCCCATCGTATTCGAGGAGTCAAGGATTTGCTGGGATTCCCCGGATC
Parent6_Nad1_A06        GCCAGGTGTGAAATCCCATCGTATTCGAGGAGTCAAGGATTTGCTGGGATTCCCCGGATC
Parent7_Nad1_A07        GCCAGGTGTGAAATCCCATCGTATTCGAGGAGTCAAGGATTTGCTGGGAATTCCCGGATC
Parent8_Nad1_A08        GCCAGGTGTGAAATCCCATCGTATTCGAGGAGTCAAGGATTTGCTGGGATTCCCCGGATC
Parent9_Nad1_A09        GCCAGGTGTGAAATCCCATCGTATTCGAGGAGTCAAGGATTTGCT---------------
Parent10_Nad1_A10       GCCAGGTGTGAAATCCCATCGTATTCGAGGAGTCAAGGATTTGCTGGGATTTCCCGGATC
Parent11_Nad1_A11       GCCAGGTGTGAAATCCCATCGTATTCGAGGAGTCAAGGATTTGCTGGGATCCCCGGATCG
Parent12_Nad1_A12       GCCAGGTGTGAAATCCCATCGTATTCGAGGAGTCAAGGATTTGCTGGGAATCCCCGGATC
                        ******************************************

Parent1_Nad1_A01        G
Parent2_Nad1_A02        G
Parent3_Nad1_A03        G
Parent4_Nad1_A04        G
Parent5_Nad1_A05        G
Parent6_Nad1_A06        G
Parent7_Nad1_A07        G
Parent8_Nad1_A08        G
Parent9_Nad1_A09        -
Parent10_Nad1_A10       G
Parent11_Nad1_A11       -
Parent12_Nad1_A12       G

Parent1_Nad3_C01        ----CAAAGGATTGTTCATGGAACTACTATCGAAATTATTCGGACCATATTTCCAAGTGT
Parent2_Nad3_C02        -------AGGATTGTTCATGGAACTACTATCGAAATTATTCGGACCATATTTCCAAGTGT
Parent3_Nad3_C03        ------AAGGATTGTTCATGGAACTACTATCGAAATTATTCGGACCATATTTCCAAGTGT
Parent4_Nad3_C04        ------AAGGATTGTTCATGGAACTACTATCGAAATTATTCGGACCATATTTCCAAGTGT
Parent5_Nad3_C05        CCCACAAAGGATTGTTCATGGAACTACTATCGAAATTATTCGGACCATATTTCCAAGTGT
Parent6_Nad3_C06        ----CAAAGGATTGTTCATGGAACTACTATCGAAATTATTCGGACCATATTTCCAAGTGT
Parent7_Nad3_C07        CCCACAAAGGATTGTTCATGGAACTACTATCGAAATTATTCGGACCATATTTCCAAGTGT
Parent8_Nad3_C08        ----CAAAGGATTGTTCATGGAACTACTATCGAAATTATTCGGACCATATTTCCAAGTGT
Parent9_Nad3_C09        ----CAAAGGATTGTTCATGGAACTACTATCGAAATTATTCGGACCATATTTCCAAGTGT
Parent10_Nad3_C10       ----CAAAGGATTGTTCATGGAACTACTATCGAAATTATTCGGACCATATTTCCAAGTGT
Parent11_Nad3_C11       CCCACAAAGGATTGTTCATGGAACTACTATCGAAATTATTCGGACCATATTTCCAAGTGT
Parent12_Nad3_C12       -------AGGATTGTTCATGGAACTACTATCGAAATTATTCGGACCATATTTCCAAGTGT
                              **************************************************

Parent1_Nad3_C01        CATTCTTTTGTTCATTGCTATACCATCGTTTGGTCTTGCGTTGACAGTACTAATAATGGC
Parent2_Nad3_C02        CATTCTTTTGTTCATTGCTATACCATCGTTTGGTCTTGCGTTGACAGTACTAATAATGGC
Parent3_Nad3_C03        CATTCTTTTGTTCATTGCTATACCATCGTTTGGTCTTGCGTTGACAGTACTAATAATGGC
Parent4_Nad3_C04        CATTCTTTTGTTCATTGCTATACCATCGTTTGGTCTTGCGTTGACAGTACTAATAATGGC
Parent5_Nad3_C05        CATTCTTTTGTTCATTGCTATACCATCGTTTGGTCTTGCGTTGACAGTACTAATAATGGC
```

```
Parent6_Nad3_C06        CATTCTTTTGTTCATTGCTATACCATCGTTTGGTCTTGCGTTGACAGTACTAATAATGGC
Parent7_Nad3_C07        CATTCTTTTGTTCATTGCTATACCATCGTTTGGTCTTGCGTTGACAGTACTAATAATGGC
Parent8_Nad3_C08        CATTCTTTTGTTCATTGCTATACCATCGTTTGGTCTTGCGTTGACAGTACTAATAATGGC
Parent9_Nad3_C09        CATTCTTTTGTTCATTGCTATACCATCGTTTGGTCTTGCGTTGACAGTACTAATAATGGC
Parent10_Nad3_C10       CATTCTTTTGTTCATTGCTATACCATCGTTTGGTCTTGCGTTGACAGTACTAATAATGGC
Parent11_Nad3_C11       CATTCTTTTGTTCATTGCTATACCATCGTTTGGTCTTGCGTTGACAGTACTAATAATGGC
Parent12_Nad3_C12       CATTCTTTTGTTCATTGCTATACCATCGTTTGGTCTTGCGTTGACAGTACTAATAATGGC
                        ************************************************************

Parent1_Nad3_C01        ATTTGTTGTCTTATTCGGAGTTCAAGGAATAGCCTTTCATCTTGGGAATGAGAATGTCGC
Parent2_Nad3_C02        ATTTGTTGTCTTATTCGGAGTTCAAGGAATAGCCTTTCATCTTGGGAATGAGAATGTCGC
Parent3_Nad3_C03        ATTTGTTGTCTTATTCGGAGTTCAAGGAATAGCCTTTCATCTTGGGAATGAGAATGTCGC
Parent4_Nad3_C04        ATTTGTTGTCTTATTCGGAGTTCAAGGAATAGCCTTTCATCTTGGGAATGAGAATGTCGC
Parent5_Nad3_C05        ATTTGTTGTCTTATTCGGAGTTCAAGGAATAGCCTTTCATCTTGGGAATGAGAATGTCGC
Parent6_Nad3_C06        ATTTGTTGTCTTATTCGGAGTTCAAGGAATAGCCTTTCATCTTGGGAATGAGAATGTCGC
Parent7_Nad3_C07        ATTTGTTGTCTTATTCGGAGTTCAAGGAATAGCCTTTCATCTTGGGAATGAGAATGTCGC
Parent8_Nad3_C08        ATTTGTTGTCTTATTCGGAGTTCAAGGAATAGCCTTTCATCTTGGGAATGAGAATGTCGC
Parent9_Nad3_C09        ATTTGTTGTCTTATTCGGAGTTCAAGGAATAGCCTTTCATCTTGGGAATGAGAATGTCGC
Parent10_Nad3_C10       ATTTGTTGTCTTATTCGGAGTTCAAGGAATAGCCTTTCATCTTGGGAATGAGAATGTCGC
Parent11_Nad3_C11       ATTTGTTGTCTTATTCGGAGTTCAAGGAATAGCCTTTCATCTTGGGAATGAGAATGTCGC
Parent12_Nad3_C12       ATTTGTTGTCTTATTCGGAGTTCAAGGAATAGCCTTTCATCTTGGGAATGAGAATGTCGC
                        ************************************************************

Parent1_Nad3_C01        GGATCTCAATGTTCTCGTCATGACCAATGCTCCTAACGGGGGTGACTTTCCCATAGACCA
Parent2_Nad3_C02        GGATCTCAATGTTCTCGTCATGACCAATGCTCCTAACGGGGGTGACTTTCCCATAGACCA
Parent3_Nad3_C03        GGATCTCAATGTTCTCGTCATGACCAATGCTCCTAACGGGGGTGACTTTCCCATAGACCA
Parent4_Nad3_C04        GGATCTCAATGTTCTCGTCATGACCAATGCTCCTAACGGGGGTGACTTTCCCATAGACCA
Parent5_Nad3_C05        GGATCTCAATGTTCTCGTCATGACCAATGCTCCTAACGGGGGTGACTTTCCCATAGACCA
Parent6_Nad3_C06        GGATCTCAATGTTCTCGTCATGACCAATGCTCCTAACGGGGGTGACTTTCCCATAGACCA
Parent7_Nad3_C07        GGATCTCAATGTTCTCGTCATGACCAATGCTCCTAACGGGGGTGACTTTCCCATAGACCA
Parent8_Nad3_C08        GGATCTCAATGTTCTCGTCATGACCAATGCTCCTAACGGGGGTGACTTTCCCATAGACCA
Parent9_Nad3_C09        GGATCTCAATGTTCTCGTCATGACCAATGCTCCTAACGGGGGTGACTTTCCCATAGACCA
Parent10_Nad3_C10       GGATCTCAATGTTCTCGTCATGACCAATGCTCCTAACGGGGGTGACTTTCCCATAGACCA
Parent11_Nad3_C11       GGATCTCAATGTTCTCGTCATGACCAATGCTCCTAACGGGGGTGACTTTCCCATAGACCA
Parent12_Nad3_C12       GGATCTCAATGTTCTCGTCATGACCAATGCTCCTAACGGGGGTGACTTTCCCATAGACCA
                        ************************************************************

Parent1_Nad3_C01        ACCTCCCGTTGGCGGACTACCAGACCTCCAGCAGGAAATGGCGCACGCCGCCCATCCCGA
Parent2_Nad3_C02        ACCTCCCGTTGGCGGACTACCAGACCTCCAGCAGGAAATGGCGCACGCCGCCCATCCCGA
Parent3_Nad3_C03        ACCTCCCGTTGGCGGACTACCAGACCTCCAGCAGGAAATGGCGCACGCCGCCCATCCCGA
Parent4_Nad3_C04        ACCTCCCGTTGGCGGACTACCAGACCTCCAGCAGGAAATGGCGCACGCCGCCCATCCCGA
Parent5_Nad3_C05        ACCTCCCGTTGGCGGACTACCAGACCTCCAGCAGGAAATGGCGCACGCCGCCCATCCCGA
Parent6_Nad3_C06        ACCTCCCGTTGGCGGACTACCAGACCTCCAGCAGGAAATGGCGCACGCCGCCCATCCCGA
Parent7_Nad3_C07        ACCTCCCGTTGGCGGACTACCAGACCTCCAGCAGGAAATGGCGCACGCCGCCCATCCCGA
Parent8_Nad3_C08        ACCTCCCGTTGGCGGACTACCAGACCTCCAGCAGGAAATGGCGCACGCCGCCCATCCCGA
Parent9_Nad3_C09        ACCTCCCGTTGGCGGACTACCAGACCTCCAGCAGGAAATGGCGCACGCCGCCCATCCCGA
Parent10_Nad3_C10       ACCTCCCGTTGGCGGACTACCAGACCTCCAGCAGGAAATGGCGCACGCCGCCCATCCCGA
Parent11_Nad3_C11       ACCTCCCGTTGGCGGACTACCAGACCTCCAGCAGGAAATGGCGCACGCCGCCCATCCCGA
Parent12_Nad3_C12       ACCTCCCGTTGGCGGACTACCAGACCTCCAGCAGGAAATGGCGCACGCCGCCCATCCCGA
                        ************************************************************

Parent1_Nad3_C01        AGACTAAATTGCGGA-
Parent2_Nad3_C02        AGACTAAATTGCGGA-
Parent3_Nad3_C03        AGACATAATTGCGGA-
Parent4_Nad3_C04        AGACTAAATTGCGGAA
Parent5_Nad3_C05        AGACTAAATTGCGGA-
Parent6_Nad3_C06        AGACTAAATTGCGGA-
Parent7_Nad3_C07        AGACAAAAATTGCGGA
Parent8_Nad3_C08        AGACATAAATTGCGGA
Parent9_Nad3_C09        AGAATAATTGCGGA--
Parent10_Nad3_C10       AGACATAAATTGCGGA
Parent11_Nad3_C11       AGACTAAATTGCGGA-
Parent12_Nad3_C12       AGACTAATTGCGGA--
                        ***   *

Parent1_Nad4_D01        ---------TCTTCTCCGATTATACGGAAGCCGGGGACGCAAGCGAATTCGTCGCAATAG
Parent2_Nad4_D02        ---------TCTTCTCCGATTATACGGAAGCCGGGGACGCAAGCGAATTCGTCGCAATAG
Parent3_Nad4_D03        --------ATCTTCTCCGATTATACGGAAGCCGGGGACGCAAGCGAATTCGTCGCAATAG
Parent4_Nad4_D04        ----------TTCTCCGATTATACGGAAGCCGGGGACGCAAGCGAATTCGTCGCAATAG
Parent5_Nad4_D05        ----------CTTCTCCGATTATACGGAAGCCGGGGACGCAAGCGAATTCGTCGCAATAG
Parent6_Nad4_D06        ----------CTTCTCCGATTATACGGAAGCCGGGGACGCAAGCGAATTCGTCGCAATAG
```

```
Parent7_Nad4_D07        ---------TCTTCTCCGATTATACGGAAGCCGGGGACGCAAGCGAATTCGTCGCAATAG
Parent8_Nad4_D08        -CTTCACAATCTTCTCCGATTATACGGAAGCCGGGGACGCAAGCGAATTCGTCGCAATAG
Parent9_Nad4_D09        ---------TCTTCTCCGATTATACGGAAGCCGGGGACGCAAGCGAATTCGTCGCAATAG
Parent10_Nad4_D10       CTTCACAAATCTTCTCCGATTATACGGAAGCCGGGGACGCAAGCGAATTCGTCGCAATAG
Parent11_Nad4_D11       -CTTCACAATCTTCTCCGATTATACGGAAGCCGGGGACGCAAGCGAATTCGTCGCAATAG
Parent12_Nad4_D12       ---------TCTTCTCCGATTATACGGAAGCCGGGGACGCAAGCGAATTCGTCGCAATAG
                                 *********************************************

Parent1_Nad4_D01        CCACCAATCTGGCGAAGCGGTTTCGCCGCGCCGAGCTCGGTGAACCGGATTCTCCCGACC
Parent2_Nad4_D02        CCACCAATCTGGCGAAGCGGTTTCGCCGCGCCGAGCTCGGTGAACCGGATTCTCCCGACC
Parent3_Nad4_D03        CCACCAATCTGGCGAAGCGGTTTCGCCGCGCCGAGCTCGGTGAACCGGATTCTCCCGACC
Parent4_Nad4_D04        CCACCAATCTGGCGAAGCGGTTTCGCCGCGCCGAGCTCGGTGAACCGGATTCTCCCGACC
Parent5_Nad4_D05        CCACCAATCTGGCGAAGCGGTTTCGCCGCGCCGAGCTCGGTGAACCGGATTCTCCCGACC
Parent6_Nad4_D06        CCACCAATCTGGCGAAGCGGTTTCGCCGCGCCGAGCTCGGTGAACCGGATTCTCCCGACC
Parent7_Nad4_D07        CCACCAATCTGGCGAAGCGGTTTCGCCGCGCCGAGCTCGGTGAACCGGATTCTCCCGACC
Parent8_Nad4_D08        CCACCAATCTGGCGAAGCGGTTTCGCCGCGCCGAGCTCGGTGAACCGGATTCTCCCGACC
Parent9_Nad4_D09        CCACCAATCTGGCGAAGCGGTTTCGCCGCGCCGAGCTCGGTGAACCGGATTCTCCCGACC
Parent10_Nad4_D10       CCACCAATCTGGCGAAGCGGTTTCGCCGCGCCGAGCTCGGTGAACCGGATTCTCCCGACC
Parent11_Nad4_D11       CCACCAATCTGGCGAAGCGGTTTCGCCGCGCCGAGCTCGGTGAACCGGATTCTCCCGACC
Parent12_Nad4_D12       CCACCAATCTGGCGAAGCGGTTTCGCCGCGCCGAGCTCGGTGAACCGGATTCTCCCGACC
                        ************************************************************

Parent1_Nad4_D01        CCGATGCGCCTTTGGAAGAGCAGGAGGCTCATTCCACGGGATCCCCTGAAAGCACAAAAG
Parent2_Nad4_D02        CCGATGCGCCTTTGGAAGAGCAGGAGGCTCATTCCACGGGATCCCCTGAAAGCACAAAAG
Parent3_Nad4_D03        CCGATGCGCCTTTGGAAGAGCAGGAGGCTCATTCCACGGGATCCCCTGAAAGCACAAAAG
Parent4_Nad4_D04        CCGATGCGCCTTTGGAAGAGCAGGAGGCTCATTCCACGGGATCCCCTGAAAGCACAAAAG
Parent5_Nad4_D05        CCGATGCGCCTTTGGAAGAGCAGGAGGCTCATTCCACGGGATCCCCTGAAAGCACAAAAG
Parent6_Nad4_D06        CCGATGCGCCTTTGGAAGAGCAGGAGGCTCATTCCACGGGATCCCCTGAAAGCACAAAAG
Parent7_Nad4_D07        CCGATGCGCCTTTGGAAGAGCAGGAGGCTCATTCCACGGGATCCCCTGAAAGCACAAAAG
Parent8_Nad4_D08        CCGATGCGCCTTTGGAAGAGCAGGAGGCTCATTCCACGGGATCCCCTGAAAGCACAAAAG
Parent9_Nad4_D09        CCGATGCGCCTTTGGAAGAGCAGGAGGCTCATTCCACGGGATCCCCTGAAAGCACAAAAG
Parent10_Nad4_D10       CCGATGCGCCTTTGGAAGAGCAGGAGGCTCATTCCACGGGATCCCCTGAAAGCACAAAAG
Parent11_Nad4_D11       CCGATGCGCCTTTGGAAGAGCAGGAGGCTCATTCCACGGGATCCCCTGAAAGCACAAAAG
Parent12_Nad4_D12       CCGATGCGCCTTTGGAAGAGCAGGAGGCTCATTCCACGGGATCCCCTGAAAGCACAAAAG
                        ************************************************************
Parent1_Nad4_D01        CAAATGATGGGGCGAACGAGGCAGGCCCGTCTTCTTCGCGGAAGCGTAAAAGGTGGGATG
Parent2_Nad4_D02        CAAATGATGGGGCGAACGAGGCAGGCCCGTCTTCTTCGCGGAAGCGTAAAAGGTGGGATG
Parent3_Nad4_D03        CAAATGATGGGGCGAACGAGGCAGGCCCGTCTTCTTCGCGGAAGCGTAAAAGGTGGGATG
Parent4_Nad4_D04        CAAATGATGGGGCGAACGAGGCAGGCCCGTCTTCTTCGCGGAAGCGTAAAAGGTGGGATG
Parent5_Nad4_D05        CAAATGATGGGGCGAACGAGGCAGGCCCGTCTTCTTCGCGGAAGCGTAAAAGGTGGGATG
Parent6_Nad4_D06        CAAATGATGGGGCGAACGAGGCAGGCCCGTCTTCTTCGCGGAAGCGTAAAAGGTGGGATG
Parent7_Nad4_D07        CAAATGATGGGGCGAACGAGGCAGGCCCGTCTTCTTCGCGGAAGCGTAAAAGGTGGGATG
Parent8_Nad4_D08        CAAATGATGGGGCGAACGAGGCAGGCCCGTCTTCTTCGCGGAAGCGTAAAAGGTGGGATG
Parent9_Nad4_D09        CAAATGATGGGGCGAACGAGGCAGGCCCGTCTTCTTCGCGGAAGCGTAAAAGGTGGGATG
Parent10_Nad4_D10       CAAATGATGGGGCGAACGAGGCAGGCCCGTCTTCTTCGCGGAAGCGTAAAAGGTGGGATG
Parent11_Nad4_D11       CAAATGATGGGGCGAACGAGGCAGGCCCGTCTTCTTCGCGGAAGCGTAAAAGGTGGGATG
Parent12_Nad4_D12       CAAATGATGGGGCGAACGAGGCAGGCCCGTCTTCTTCGCGGAAGCGTAAAAGGTGGGATG
                        ************************************************************
Parent1_Nad4_D01        ATGATTCGGGGTCCGAAGATGATGATTCGGGGGGCCCCGGGGAATCTCCAGATCCAGTGG
Parent2_Nad4_D02        ATGATTCGGGGTCCGAAGATGATGATTCGGGGGGCCCCGGGGAATCTCCAGATCCAGTGG
Parent3_Nad4_D03        ATGATTCGGGGTCCGAAGATGATGATTCGGGGGGCCCCGGGGAATCTCCAGATCCAGTGG
Parent4_Nad4_D04        ATGATTCGGGGTCCGAAGATGATGATTCGGGGGGCCCCGGGGAATCTCCAGATCCAGTGG
Parent5_Nad4_D05        ATGATTCGGGGTCCGAAGATGATGATTCGGGGGGCCCCGGGGAATCTCCAGATCCAGTGG
Parent6_Nad4_D06        ATGATTCGGGGTCCGAAGATGATGATTCGGGGGGCCCCGGGGAATCTCCAGATCCAGTGG
Parent7_Nad4_D07        ATGATTCGGGGTCCGAAGATGATGATTCGGGGGGCCCCGGGGAATCTCCAGATCCAGTGG
Parent8_Nad4_D08        ATGATTCGGGGTCCGAAGATGATGATTCGGGGGGCCCCGGGGAATCTCCAGATCCAGGGG
Parent9_Nad4_D09        ATGATTCGGGGTCCGAAGATGATGATTCGGGGGGCCCCGGGGAATCTCCAGATCCAGTGG
Parent10_Nad4_D10       ATGATTCGGGGTCCGAAGATGATGATTCGGGGGGCCCCGGGGAATCTCCAGATCCAGTGG
Parent11_Nad4_D11       ATGATTCGGGGTCCGAAGATGATGATTCGGGGGGCCCCGGGGAATCTCCAGATCCAGTGG
Parent12_Nad4_D12       ATGATTCGGGGTCCGAAGATGATGATTCGGGGGGCCCCGGGGAATCTCCAGATCCAGGGG
                        ************************************************************ **

Parent1_Nad4_D01        GAAATCGT-
Parent2_Nad4_D02        AAATCGT--
Parent3_Nad4_D03        AAATCGTA-
Parent4_Nad4_D04        GAAATCGTA
Parent5_Nad4_D05        GAAATCGT-
```

```
Parent6_Nad4_D06        GAAATCGTA
Parent7_Nad4_D07        AAAT-----
Parent8_Nad4_D08        AAATCGTA-
Parent9_Nad4_D09        AAATCGT--
Parent10_Nad4_D10       AAATCGTA-
Parent11_Nad4_D11       GAAATCGTA
Parent12_Nad4_D12       AAATCG---
                          **
```

First, we sequenced gene *nad*3 in eight Australian hexaploid and four Australian durum wheat lines. The consensus sequences for the wheat mitochondrial gene *nad*3 were obtained from the Genebank accession X59153.1 (Gualberto et al. 1991) and provided in Supplementary Table -1. Alternatively, we used the consensus sequences of targeted mitochondrial sequences of hexaploid bread wheat (*Triticum aestivum* L.) cultivar Chinese Spring (Genbank accession X59153.1, EU 534409.1 and GU9855444.1) and tetraploid durum wheat *T. turgidium* spp. *durum* (Genbank accession KJ078649.1) obtained from the NCBI website to design primers for *nad*3, *ccmfc* and *ccmfn*. Details of the targeted regions that were used in the present study are given in the Supplementary Figure -1. Primers to test the mitochondrial regions, including *nad*3, *ccmfn*, and *ccmfc* were designed using PRIMER3 software based on the mitochondrial genome sequences obtained from the NCBI website (Table-1).

Table -1 Primer sequences used for the amplification of targeted mt DNA of bread wheat.

Genes	Forward	Reverse	Product size	Annealing temperature
nad 3 -1	ATGAATGGAAAAGGGGTGCTT	TTTCCCTTCTAGATTTATACC	432	58
nad 3-3	GGTTCGCGCTTTATGGCATT	TGAAATATTCTTTCAACCTT	387	60
nad 3 -4	GGGGATGCACAAGGGTACAA	CCATTCCTCGTGAGCCACTT	497	55
ccmfn	CTTCCTGGCGAGATGCCTAC	TTCCTTGCCGCTGAGACTGA	959	60
Ccmfc-1	CCCCCAAAAACAAACCGACC	GAACGGGCTTCCCAAAAAGC	355	60
Ccmfc-2	AAAGCCGCCGAAGCAGCAT	TCAGCTATACATCCTCCTT	280	60
Ccmfc-3	AAGGGAGTGTACAACTGG	TTACAACGTATTAACCGCAT	270	57

Total genomic DNA extraction and amplification of mt DNA regions

Total DNA was extracted from three-week-old leaves using a Wizard Genomic DNA Purification Kit (Promega Corporation, Sydney, Australia) as per the manufacturer's

instructions. A standard 25µl polymerase chain reaction (PCR) was performed with designed primers of targeted mt regions (Table-1) using MyTaq™ DNA polymerase. Of the 25µl PCR product, a 5µl aliquot was electrophoresed on a 1% agarose gel. Amplified PCR products were stained using Gel Red (Biotium, Sydney Australia) and visualized on a Biorad XR+ gel documentation system.

DNA sequencing and analysis

The remaining 20µl of the PCR product was purified, using a Sure Clean Plus kit (Bioline, Sydney, Australia). Sanger sequencing was performed using the service provided by the Australian Genome Research Facility Ltd (AGRF) and Macrogen online sequencing (Republic of Korea). Sequencing results were assessed using Geneious software version 10.2.3. If any SNP were identified between bread and durum wheat cultivars KASP™ (kompetitive allele-specific PCR) markers were used to validate the nucleotide difference.

KASP assay

KASP™ assay was performed using a KASP master mix (www.lgcgroup.com) with a designed SNP marker that contains two forward primers and one common reverse primer Table -3. Both forward primers contain an additional tail sequence and each contained a unique VIC® and FAM® FRET (fluorescence resonance energy transfer). The PCR cocktail was made to perform a 10µl reaction including targeted DNA samples. Samples were amplified using a Rotor-Gene 6000 (Qiagen, Sydney, Australia). The amplified PCR products were also loaded onto a 0.6% polyacrylamide gel using the GEL scan 2000 (Corbett Research-Sydney Australia) and stained with 5µl Gel Red (Biotium, Sydney Australia). The results were scored 1 and 0 based on the presence or absence of the amplified PCR product.

Results

Mitochondrial Gene nad3

The mitochondrial gene *nad*3 was sequenced in eight Australian bread wheat and four Australian durum wheat cultivars to determine whether there are any differences in the

nucleotide sequences. The resultant sequences from the AGRF were aligned between bread and durum wheat cultivars and are shown in Figure-1 and the raw sequences obtained from the NCBI website were given in the Supplementary Figure-1. The results indicated that there was a difference within the bread wheat cultivars. However, we could not distinguish any differences between bread and durum wheat. Therefore we decided to look into cytochrome maturation *ccmfn*, *ccmfc* genes of the mitochondria.

Nucleotide differences in mt DNA between bread and durum wheat

Whole mitochondrial targeted gene sequences of bread and durum wheat were accessed from the NCBI website to identify nucleotide differences. Most of the mt DNA regions including (*nad*-1, 3, 5, 6, *Cox*-1, 2, 3, *Ccm fc Ccm fn* and open reading frames (*Orf*) 173, 240, 349) were matching between bread and durum wheat (Table -2). The aligned sequences of targeted genes of bread and durum wheat are presented in the Supplementary Figure-2. Differences were identified in the cytochrome maturation regions; *ccmfn* and *ccmfc* (Supplementary Figure-2) and these were considered for analysis in the present study. The size of the *ccmfn* and *ccmfc* genes were 2320 and 520bp respectively. The *ccmfn* gene of durum wheat had one bp deletion at 130421 bps and four transversions from 130681 to 131684 bps (G>T, A>C, A>C, and A>T). Whereas the *ccmfc* gene of durum wheat had one transversion at 10826 bps (A>C) when compared to bread wheat.

Table -2 Nucleotide differences identified between the bread and durum wheat based on the sequences obtained from NCBI

Genes	*Triticum aestivum* (GU985444.1)		*T. turgidium* sp. *durum* (KJ078650.1)		Size (bp)	No of SNP's	Nucleotide differences
	Start	End	Start	End			
nad-1	226434	226819	61950	62335	386	0	
nad-3	181883	182239	343021	343377	356	0	
nad-5	323719	323949	376970	377200	230	0	
nad-6	338493	339182	361733	362422	689	0	
cox-1	265395	266965	301219	302789	1571	1	1-Transversion
	543217	543606	301995	302384	390	0	
cox-2	544833	545225	320333	320854	393		
cox-3	85376	86173	70264	71061	797	2	2-Transversion

Ccm fc	22053	24373	129160	131479	2320	5	1-Deletion 4-Transversion
Ccm fn	213152	213672	10793	11313	520	1	1-Transversion
Orf-173	561548	562069	320333	320854	522	0	
Orf-240	70866	71588	84870	85592	722	1	1-Transition
Orf-349	554801	555850	313579	314628	1050	1	1-Transversion

Primer amplifications

Since the size of the *ccmfc* gene is 2320bps, it is too large to sequence in one go. Thus, we designed two pairs of primers amplifying the region where the SNP was detected (Table 1). With regards to the *ccmfn* gene, a set of primers were designed for this gene which includes 200 bps before and after the gene. The primers were used to amplify the parental bread and durum wheat selected lines and the size of the amplicon was verified. The purified and cleaned amplicon was subjected to Sanger sequencing.

Unfortunately, AGRF reported that samples of the *ccmfn* amplicons did not pass their quality check due to the low and degraded DNA quality. Thus Sanger sequencing was not performed on these samples. Sanger sequencing was performed on the remaining *ccmfc* samples except for bread wheat line LRC2010-150 which did not produce a quality sequence. The resultant forward and reverse sequences of the *ccmfc* regions belonged to two bread (Sunguard and 2-49) and three durum wheat lines (Bellaroi, WID-802 and Parent B (950329) and each indicated nucleotide differences across the two different species. Furthermore, the results also indicated that there are SNP's within the hexaploid and tetraploid cultivars (Figure-2).

Figure-2 Sanger sequencing alignment for *ccmfc* gene for two bread and three durum wheat cultivars.

```
1_2SUNGUARD_F_A02       ---AGCGCTCATCCCTTGCTTGCTGCTTCGCGTTCTTTCTATTCCATCCCAGTCCATTCC
1_32_49_F_A03           ---AGCGCTCATCCCTTGCTTGCTGCTTCGCGTTCTTTCTATTCCATCCCAGTCCATTCC
1_5PARENTB_F_A05        ACAAGCGCTCATCCCTTGCTTGCTGCTTCGCGTTCTTTCTATTCCATCCCAGTCCATTCC
1_6BELLAROI_F_A06       ---AGCGCTCATCCCTTGCTTGCTGCTTCGCGTTCTTTCTATTCCATCCCAGTCCATTCC
1_4WID802_F_A04         ACAAGCGCTCATCCCTTGCTTGCTGCTTCGCGTTCTTTCTATTCCATCCCAGTCCATTCC
                           ************************************************************

1_2SUNGUARD_F_A02       GGGATAGGCGGCTAATAGAATCTAATATGTGCAGTACTCGTCGTCTGACCAATCGGCTCG
1_32_49_F_A03           GGGATAGGCGGCTAATAGAATCTAATATGTGCAGTACTCGTCATCTGACCAATCGGCTCG
1_5PARENTB_F_A05        GGGATAGGCGGCTAATAGAATCTAATATGTGCAGTAGTCGTCGTCTGACCAATCGGCTCG
1_6BELLAROI_F_A06       GGGATAGGCGGCTAATAGAATCTAATATGTGCAGTAGTCGTCGTCTGACCAATCGGCTCG
1_4WID802_F_A04         GGGATAGGCGGCTAATAGAATCTAATATGTGCAGTAGTCGTCGTCTGACCAATCGGCTCG
                        ********************************** ***** ****************

1_2SUNGUARD_F_A02       GACACCAAACCACTTGTGCCCGCCCATTCTGTCTCGCCCTAAATGGAATGGCTCTCTTAG
1_32_49_F_A03           GACACCAAACCACTTGTGCCCGCCCATTCTGTCTCGCCCTAAATGGAATGGCTCTCTTAG
```

```
1_5PARENTB_F_A05      GACACCAGACCACTTGTGCCCGCCCATTCTGTCTCGCCCTAAATGGAATGGCTCTCTTAG
1_6BELLAROI_F_A06     GACACCAGACCACTTGTGCCCGCCCATTCTGTCTCGCCCTAAATGGAATGGCTCTCTTAG
1_4WID802_F_A04       GACACCAGACCACTTGTGCCCGCCCATTCTGTCTCGCCCTAAATGGAATGGCTCTCTTAG
                      ******* **************************************************

1_2SUNGUARD_F_A02     TTACGCTGTGCCCCGACCCGAGTCCCCACGTCCGCTTTTATCCGCCCGCAACCCGGCCAA
1_32_49_F_A03         TTACGCTGTGCCCCGACCCGAGTCCCCACGTCCGCTTTTATCCGCCCCCAACCCGGCCAA
1_5PARENTB_F_A05      TTACGCTGTGCCCCGACCCGAGTCCCCACGTCCGCTTTTATCCGCCCGCAACCCGGCCAA
1_6BELLAROI_F_A06     TTACGCTGTGCCCCGACCCGAGTCCCCACGTCCGCTTTTATCCGCCCGCAACCCGGCCAA
1_4WID802_F_A04       TTACGCTGTGCCCCGACCCGAGTCCCCACGTCCGCTTTTATCCGCCCGCAACCCGGCCAA
                      ********************************************* ************

1_2SUNGUARD_F_A02     CACAACATTAGGGCCGCCCCCCCCATTCTATGCTGACCCGGGCCGG--------------
1_32_49_F_A03         CACAACATTAGGGCCCCCCCCCCCATTCTATGCTGACCCGGGCCGGGGTT----------
1_5PARENTB_F_A05      CACAACATTAGGGCCGTCCCCCCCATTCTATGCTGACCCGGGCCGGGGCTCGCTTTTTGG
1_6BELLAROI_F_A06     CACAACATTAGGGCCGTCCCCCCCATTCTATGCTGACCCGGGCCGGGGCTCGCTTTTTGG
1_4WID802_F_A04       CACAACATTAGGGCCGTCCCCCCCATTCTATGCTGACCCGGGCCGGGGCTCGCTTTTTGG
                      ***************  ******************************

1_2SUNGUARD_F_A02     -----------
1_32_49_F_A03         -----------
1_5PARENTB_F_A05      GAGCCCGTTCA
1_6BELLAROI_F_A06     AAGCCC------
1_4WID802_F_A04       GAAGCCCG---

2_32_49_F_A09         ------GAGCAGGCTTCTATTGCTACGCAACAATAGAGCAGGCGCGCCGCCCACAAAATG
2_2SUNGUARD_F_A08     CTGCATGAGCAGGCTTCTATTGCTACGCAACAATAGAGCAGGCGCGCCGCCCACAAAATG
2_5PARENTB_F_A11      --GCATGAGCAGGCTTCTATTGCTACGCAACAATAGAGCAGGCGCGCCGCCCACAAAATG
2_4WID802_F_A10       ------GAGCAGGCTTCTATTGCTACGCAACAATAGAGCAGGCGCGCCGCCCACAAAATG
2_6BELLAROI_F_A12     --GCATGAGCAGGCTTCTATTGCTACGCAACAATAGAGCAGGCGCGCCGCCCACAAAATG
                            ****************************************************

2_32_49_F_A09         TTTGAATGATCGGGTAAAGAGCGAGCTTCTTATATGGGATCCGACGCATCCAGCAGAGCG
2_2SUNGUARD_F_A08     TTTGAATGATCGGGTAAAGAGCGAGCTTCTTATATGGGATCCGACGCATCCAGCAGAGCG
2_5PARENTB_F_A11      TTTGAAAGATCGGGTAAAGAGCGAGCTTCTTATATGGGATCCGACGCATCCAGCAGAGCG
2_4WID802_F_A10       TTTGAATGATCGGGTAAAGAGCGAGCTTCTTATATGGGATCCGACGCATCCAGCAGAGCG
2_6BELLAROI_F_A12     TTTGAATGATCGGGTAAAGAGCGAGCTTCTTATATGGGATCCGACGCATCCAGCAGAGCG
                      ****** ****************************************************

2_32_49_F_A09         AAGCAGCGTTCCATTCTTTTCGGCGGCATCCTTCCGCATTGGCGGCGAGTGGAGTGCCAC
2_2SUNGUARD_F_A08     AAGCAGCGTTCCATTCTTTTCGGCGGCATCCTTCCGCATTGGCGGCGAGTGGAGTGCCAC
2_5PARENTB_F_A11      AAGCAGCGTTCCATTCTTTTCGGCGGCATCCTTCCGCATTGGCGGCGAGTGGAGTGCCAC
2_4WID802_F_A10       AAGCAGCGTTCCATTCTTTTCGGCGGCATCCTTCCGCATTGGCGGCGAGTGGAGTGCCAC
2_6BELLAROI_F_A12     AAGCAGCGTTCCATTCTTTTCGGCGGCATCCTTCCGCATTGGCGGCGAGTGGAGTGCCAC
                      ************************************************************

2_32_49_F_A09         AATCCCATTCATCATTTTTGATCTACATAACCCAAAGCCCATAGCACTGGCGACATCTCC
2_2SUNGUARD_F_A08     AATCCCATTCATCATTTTTGATCTACATAACCCAAAGCCCATAGCACTGGCGACATCTCC
2_5PARENTB_F_A11      AATCCCATTCATCATTTTTGATCTACATAACCCAAAGCCCATAGCACTGGCGACATCTCC
2_4WID802_F_A10       AATCCCATTCATCATTTTTGATCTACATAACCCAAAGCCCATAGCACTGGCGACATCTCC
2_6BELLAROI_F_A12     AATCCCATTCATCATTTTTGATCTACATAACCCAAAGCCCATAGCACTGGCGACATCTCC
                      ************************************************************

2_32_49_F_A09         GGCATA----
2_2SUNGUARD_F_A08     GGGCATA---
2_5PARENTB_F_A11      GGCATA----
2_4WID802_F_A10       GGCATA----
2_6BELLAROI_F_A12     GGCATAAAATG
                      **

3_32_49_F_B03           TTTTTTGTTGGAGAGATAGAATGGAGTTCTTCACGAAGTTCGAGACAAAGGAAAAAAT
3_2SUNGUARD_F_B02       --TTTTGTTGGAGAGATAGAATGGAGTTCTTCACGAAGTTCGAGACAAAGGAAAAAAT
3_5PARENTB_F_B05          TTTTGTTGGAGAGATAGAATGGAGTTCTTCACGAAGTTCGAGACAAAGAGAAAAAT
3_4WID802_F_B04         ------GTTGGAGAGATAGAATGGAGTTCTTCACGAAGTTCGAGACAAAGGAAAAAAT
3_6BELLAROI_F_B06       --TTTTGTTGGAGAGATAGAATGGAGTTCTTCACGAAGTTCGAGACAAAGGAAAAAAT
                              ************************************************

3_32_49_F_B03         CAAAGTTTCTCTATAGCCTCTTCGTTTTGAGACATTATGGCTTTGGGGTCGACCCCGGTA
3_2SUNGUARD_F_B02     CAAAGTTTCTCTATAGCCTCTTCGTTTTGAGACATTATGGCTTTGGGGTCGACCCCGGTA
```

```
3_5PARENTB_F_B05        CAAAGTTTCTCTATAGCCTCTTCGTTTTGAGACATTATGGCTTTGGGGTCGACCCCGGTA
3_4WID802_F_B04         CAAAGTTTCTCTATAGCCTCTTCGTTTTGAGACATTATGGCTTTGGGGTCGACCCCGGTA
3_6BELLAROI_F_B06       CAAAGTTTCTCTATAGCCTCTTCGTTTTGAGACATTATGGCTTTGGGGTCGACCCCGGTA
                        ************************************************************

3_32_49_F_B03           ACAAAAAAGGAATCCATAAAAACTGGGGATCCAACACCATGATAAAATACTACCCTCATG
3_2SUNGUARD_F_B02       ACAAAAAAGGAATCCATAAAAACTGGGGATCCAACACCATGATAAAATACTACCCTCATG
3_5PARENTB_F_B05        ACAAAAAAGGAATCCATAAAAACTGGGGATCCAACACCATGATAAAATACTACCCTCATG
3_4WID802_F_B04         ACAAAAAAGGAATCCATAAAAACTGGGGATCCAACACCATGATAAAATACTACCCTCATG
3_6BELLAROI_F_B06       ACAAAAAAGGAATCCATAAAAACTGGGGATCCAACACCATGATAAAATACTACCCTCATG
                        ************************************************************

3_32_49_F_B03           ATTAGACCATGTCCCTGAGATTTGATAAAAAAA----------------------
3_2SUNGUARD_F_B02       ATTAGACCATGTCCCTGAGATTTGATAAAAAAAAGGTGCAT--------------
3_5PARENTB_F_B05        ATTAGACCATGTCCCTGAGATTTGATAAAAAAAAGGTGCATTA------------
3_4WID802_F_B04         ATTAGACCATGTCCCTGAGATTTGATAAAAAAAAGGTGCATTAGCGGGTTAATA
3_6BELLAROI_F_B06       ATTAGACCATGTCCCTGAGATTTGATAAAAAAAAGG------------------
                        ********************************
```

KASP assay

Based on the Sanger sequencing results, SNP markers were designed for the polymorphic regions and the primer sequences are presented in Table-3. The KASP assay failed to distinguish SNPs between the bread and durum wheat parents as the same fragment was amplified across all lines. Since the KASP assays failed, we re-amplified the *ccmfc* region in both parents and 20 progenies derived from hexaploid/tetraploid and tetraploid/ hexaploid crosses and had them sequenced using the Macrogen (Seoul, South Korea) sequencing facility. This alignment of the sequences for all lines confirmed that the sequences that were generated on the targeted gene. However, the sequences generated were of poor quality with Q values (quality value) of less than 10. Since the majority of the data peaks were of poor quality across the parents and the progenies, the SNP's could not be clearly distinguished between hexaploid/tetraploid hybrids.

Table-3 SNP markers sequences used for the amplification of *ccmfc* gene –KASP assay

Discussion

Genbank Accession	Forward-VIC	Forward-FAM	Reverse	Annealing temperature	Size of amplicon
KJ078650.1 130009-130750	GCTAATATAATATGTGCAGTAC	GCTAATATAATATGTGCAGTAG	TAGGGCGAGACAGAATGG	60	88
KJ078650.1 130180-130500	GACCAATCGGCTCGGACACCAA	GACCAATCGGCTCGGACACCAG	GGGGCACAGCGTAACTAAGA	55	88
KJ078650.1 130180-130500	GCCAACACAACATTAGGGCCGC	GCCAACACAACATTAGGGCCGT	ACGGGCTTCCCAAAAAGC	60	74
KJ078650.1 129800-130100	CCAGCAAGAAAACGTATGCGCT	CCAGCAAGAAAACGTATGCGCA	GGAATGGACTGGGATGGAAT	60	89

This is the first study aiming to test the maternal inheritance of mitochondrial DNA sequences in interploidy crosses using a pair of reciprocal hexaploid/tetraploid and tetraploid/hexaploid crosses. Inter or intra-ploidy derived progenies often have issues related to poor seed set and often are infertile in subsequent generations (Bhagyalakshmi et al. 2008). This poor seed set or infertile nature of inter or intra ploidy hybrids is often associated with cytoplasmic male sterility (CMS) and has been reported in >150 different plant species (Leon et al. 1998). Nucleotide rearrangement of mt DNA genes under the control of nuclear fertility restorer genes is one of the major causes for the expression of CMS (MacKenzie et al. 1988).

We identified a nucleotide difference in a known cytochrome maturation gene *ccmfn* that is consistent across four Australian bread wheat and three Australian durum wheat cultivars. Recent literature has proved that there are more than 40 nucleotide differences (SNP's) across the whole mitochondrial genome between these two ploidy level wheat species (Noyszewski et al. 2014). These previous investigations compared nucleotide differences in the mitochondrial sequences of bread wheat cultivar Chinese Spring with an unknown durum line (Noyszewski et al., 2014) and also compared between Chinese Yumai (Hexaploid) and Chinese Spring (Hexaploid) (Cui et al. 2009).

Clearly, previous investigations have not screened enough bread and durum wheat cultivars to determine whether previously identified SNPs are true across multiple genotypes. Furthermore, from this investigation, it is clear that there are some nucleotide differences in the major mitochondrial genes within the bread and durum wheat cultivars. However, this needs to be validated by specific SNP markers that can distinguish the polymorphic differences between current bread or durum wheat cultivars, since whole mitochondrial genome assemblies are available for the two oldest wheat cultivars (Chinese Spring and Yumai) and their genome assemblies may be different to the modern bread wheat cultivars.

Detection of specific nucleotide differences between the bread and durum wheat mt DNA fragments might help to develop a marker that can distinguish these two ploidy level wheat species. This mitochondrial DNA marker could be used to trace the inheritance of mt DNA fragments. Sequencing of the *ccmfn* gene region containing the SNP differences between the bread and durum wheat cultivars identified three nucleotide differences. However poor sequencing results did not allow us to identify the SNP in the hexaploid/tetraploid and its reciprocal tetraploid/hexaploid progenies.

The poor quality of the sequences could be due to the low concentration of the mt DNA present in comparison to the total genomic DNA extracted (Notsu et al. 2002). Removal of the nucleus genomic DNA from the PCR amplicon, thus only isolating mt DNA, might help to improve the quality of the sequence. This could be achieved by excising the PCR fragment from the agarose gel and sending this for sequencing instead of cleaning the remaining PCR product (Glenn and Schable 2005) Unfortunately time restrains did not permit us to continue this work under the current Ph.D. study.

Although this study is still in the initial phase, we identified nucleotide differences between mt DNA of bread and durum wheat lines. These results need to be further evaluated using different bread and durum wheat mt DNA regions/genes with next-generation sequencing technique. This could help to identify a number of unique SNP markers across the whole mt genome. The SNP markers would assist in studying how the mitochondrial DNA inherits in interspecific wheat hybrids. Understanding the cytoplasmic inheritance could give insights into issues that hinder the growth and development of interploidy wheat progenies (Padmanaban et al. 2017).

References

Aksyonova E., Sinyavskaya M., Danilenko N., Pershina L., Nakamura C., Davydenko O. 2005 Heteroplasmy and paternally oriented shift of the organellar DNA composition in barley–wheat hybrids during backcrosses with wheat parents. *Genome*. **48**, 761-769.

Bhagyalakshmi K., Vinod KK., Kumar M., Arumugachamy S., Prabhakaran A., Raveendran T.S. 2008 Interspecific hybrids from wild x cultivated Triticum crosses-A study on the cytological behaviour and molecular relations. *J. Crop. Sci. Biotech*. **11**, 257-262.

Birky C.W. 1995 Uniparental inheritance of mitochondrial and chloroplast genes: mechanisms and evolution. *Proc. Natl. Acad. Sci. U.S.A*. **92**, 11331-11338.

Birky C.W. 2001 The inheritance of genes in mitochondria and chloroplasts: laws, mechanisms, and models. *Annu. Rev. Genet*. **35**, 25-148.

Bonen L., Bird S. 1988 Sequence analysis of the wheat mitochondrial *atp*6 gene reveals a fused upstream reading frame and markedly divergent N termini among plant *ATP*6 proteins. *Gene*. **73,** 47-56.

Calixte S., Bonen L. 2008 Developmentally-specific transcripts from the *ccmFN-rps*1 locus in wheat mitochondria. *Mol. Gent. Genomics*. **280**, 419-426.

Cannino G., Di Liegro C., Rinaldi A. 2007 Nuclear–mitochondrial interaction. *Mitochondrion*. **7,** 359-366.

Chapdelaine Y., Bonen L. 1991 The wheat mitochondrial gene for subunit I of the *NADH* dehydrogenase complex: A trans-splicing model for this gene-in-pieces. *Cell*. **65**, 465-472.

Cui P., Liu H., Lin Q., Ding F., Zhuo G., Hu S., Liu D., Yang W., Zhan K., Zhang A. 2009 A complete mitochondrial genome of wheat (*Triticum aestivum* cv. Chinese Yumai), and fast evolving mitochondrial genes in higher plants. *J. Genet*. **88**, 299-307.

Glenn TC, Schable N.A. 2005 Isolating Microsatellite DNA Loci. *Methods. Enzymol*. **395**, 202-222.

Gualberto JM., Bonnard G., Lamattina L., Grienenberger J.M. 1991 Expression of the wheat mitochondrial *nad*3-*rps*12 transcription unit: correlation between editing and mRNA maturation. *The Plant Cell.* **3**, 1109-1120.

Hattori N, Kitagawa K, Takumi S, Nakamura C (2002) Mitochondrial DNA heteroplasmy in wheat, Aegilops and their nucleus-cytoplasm hybrids. *Genetics* 160 (4):1619-1630

Kitagawa K, Takumi S, Nakamura C (2003) Selective transcription and post-transcriptional processing of the heteroplasmic mitochondrial *orf*156 copies in the nucleus-cytoplasm hybrids of wheat. *Plant. Mol. Biol*. **53**, 609-619.

Kmiec B., Woloszynska M., Janska H. 2006 Heteroplasmy as a common state of mitochondrial genetic information in plants and animals. *Curr. Genet*. **50**, 149-159.

Laser B., Mohr S., Odenbach W., Oettler G., Kück U. 1997 Parental and novel copies of the mitochondrial orf25 gene in the hybrid crop-plant triticale: predominant transcriptional expression of the maternal gene copy. *Curr. Genet*. **32**, 337-347.

Leon P., Arroyo A., Mackenzie S. 1998 Nuclear control of plastid and mitochondrial development in higher plants. *Annu. Rev. Plant. Biol*. **49**, 453-480.

Liu H, Cui P, Zhan K, Lin Q, Zhuo G, Guo X, Ding F, Yang W, Liu D, Hu S, Yu J, Zhang A (2011) Comparative analysis of mitochondrial genomes between a wheat K-type cytoplasmic male sterility (CMS) line and its maintainer line. BMC Genomics **12**, 163.

MacKenzie SA, Pring D, Bassett M, Chase C (1988) Mitochondrial DNA rearrangement associated with fertility restoration and cytoplasmic reversion to fertility in cytoplasmic male sterile Phaseolus vulgaris L. Proceedings of the National Academy of Sciences 85 (8):2714-2717

Miyamura S, Kuroiwa T, Nagata T (1987) Disappearance of plastid and mitochondrial nucleoids during the formation of generative cells of higher plants revealed by fluorescence microscopy. *Protoplasma*. **141**, 149-159.

Nagata N. 2010 Mechanisms for independent cytoplasmic inheritance of mitochondria and plastids in angiosperms. *J. plant. Res.* **123**, 193-199.

Notsu Y., Masood S., Nishikawa T., Kubo N., Akiduki G., Nakazono M., Hirai A., Kadowaki K. 2002 The complete sequence of the rice (*Oryza sativa* L.) mitochondrial genome: frequent DNA sequence acquisition and loss during the evolution of flowering plants. *Mol. Genet. Genomics* **268,** 434-445.

Noyszewski AK., Ghavami F., Alnemer LM., Soltani A., Gu YQ., Huo N., Meinhardt S., Kianian PM., Kianian S.F. 2014 Accelerated evolution of the mitochondrial genome in an alloplasmic line of durum wheat. *BMC. Genomics***. 15**, 67.

Padmanaban S., Zhang P., Hare RA., Sutherland MW., Martin A. 2017 Pentaploid Wheat Hybrids: Applications, Characterisation, and Challenges. *Front. Plant. Sci* **8**, 358.

Reboud X., Zeyl C. 1994 Organelle inheritance in plants. *Heredity*. **72,** 132-140.

Sato M., Sato K. 2013 Maternal inheritance of mitochondrial DNA by diverse mechanisms to eliminate paternal mitochondrial DNA. Biochimica et Biophysica Acta (BBA). *Mol. Cell. Res*. **1833** (8):1979-1984.

Whatley J.M. 1982 Ultrastructure of plastid inheritance: green algae to angiosperms. *Biol. Rev*. **57**, 527-569.

Supplementary Figure-1 Raw sequences for *nad*-3, *rps* 12 and *cox*11 obtained from the GenBank Accession: X59153.1

Wheat mitochondrial *nad*3 gene, *rps*12 gene, *ORF156* and *COXII* pseudogene for *NADH* dehydrogenase subunit 3, ribosomal protein S12, and an unknown (18kDA) membrane bound protein

GenBank: X59153.1

>gi|433681|emb|X59153.1| Wheat mitochondrial nad3 gene, rps12 gene, ORF156 and COXII pseudogene for NADH dehydrogenase subunit 3, ribosomal protein S12, and an unknown (18kDA) membrane bound protein

GAGAGCGAGAGAACGAAGTGGGCTTTGGTGATGTCGGAATTTGCACCTATTTGTATCTATTTAGTGATCA

GTCCGCTAGTTTCTTTGATTCCACTCGGTGTTCCTTTTCCATTTGCTTCCAATAGTTCGACCTATCCAGA

AAAATTGTCGGCCTACGAATGTGGTTCCGATCCCTCCGGTGATGCCAGAAGTCGTTTCGATATACGATTT

TATCTGGTTCCTATTTTATTTATTATCCCTGATCCGGAAGTCACCTTTTCTTTTCCTTGGGCAGTACCTC

CTAACAAGATTGATCTGTTTGGATCTTGGTCCATGATGGCCTTTTTATTGATTTTGACGATTGGATCTCT

CTATGAATGGAAAAGGGGTGCTTCGGATCGGGAGTAACCACTTTAGAAAGGGCAAAGGGGGGAAGGACAT

AGGAAAGAGGGATGCCTACAAAAAATCAATTGATTCGTCATGGTAGAGAAGAAAAACGGCGCACGGACCG

TACTCGAGCTTCGGATCAATGTCCCCAGAAGCAAGGAGTATGCCTGCGTGTTTCGACGAGAACACCGAAA

AAACCTAATTCAGCTCTACGTAAGATAGCAAAAGTACGGTTGAGCAATCGACATGATATATTTGCTCACA

TTCCAGGCGAAGGTCATAATTCGCAGGAACATTCTATAGTCTTAGTCAGAGGAGGTAGAGTGAAAGATTC

GCCAGGTGTGAAATCCCATCGTATTCGAGGAGTCAAGGATTTGCTGGGAATTCCGGATCGTAGAAAGGGA

AGATCTAAATATGGTGCAGAAAGACCTAAATCGAAATGAATGGAAGATGCCTCTGGAACTTTTGGGTTCT

TTTTTGGGGGCGATATGGAAGCAGCTAGCTCCCTTTCCCTTATTACGTTACCATTTCTCTCCGCTATTCT

TCGCAAATTTCATTGAGAGGAATCAGCAAAGAAAAGAAATCTTCTCCGGGTGACAGGGCCGCCCCGGCAG

CGTGGCTTCTCTGCCGAGCAGGAAGCCACAAAAAGCATTGTGCTCTTGTAACTGATCTTTTAGTAGGCTA

AGTTGCCGAATTCTGAAATCACTACAGGCACAGTGCAGGAAATTGTGGTCCTCAAAACAATGTGTCCCCA

TGATCATTTTACATCACGATATCTTTTTCTTCCTCATTCTTATTTTGGTTTTCGTATCACGGATGTTGGT

TCGCGCTTTATGGCATTTCAACGAGCAAACTAATCCAATCCCACAAAGGATTGTTCATGGAACTACTATC

GAAATTATTCGGACCATATTTCCAAGTGTCATTCTTTTGTTCATTGCTATACCATCGTTTGGTCTTGCGT

TGACAGTACTAATAATGGCATTTGTTGTCTTATTCGGAGTTCAAGGAATAGCCTTTCATCTTGGGAATGA

GAATGTCGCGGATCTCAATGTTCTCGTCATGACCAATGCTCCTAACGGGGGTGACTTTCCCATAGACCAA

CCTCCCGTTGGCGGACTACCAGACCTCCAGCAGGAAATGGCGCACGCCGCCCATCCCGAAGACATAATTG

CGGAGTTGACTTCCAAGGTTGAAAGAATATTTCACGAAGTAGGCACTCCTCTTCCTTTAGAGGAAGGTGA

ATCTGCGCGATCGTTCACTGAAAATCATGTATTGTGGAATAGTGAGGGAGGAGATACTCTTCAACAAATC

TTCTCCGATTATACGGAAGCCGGGGACGCAAGCGAATTCGTCGCAATAGCCACCAATCTGGCGAAGCGGT

TTCGCCGCGCCGAGCTCGGTGAACCGGATTCTCCCGACCCCGATGCGCCTTTGGAAGAGCAGGAGGCTCA

TTCCACGGGATCCCCTGAAAGCACAAAAGCAAATGATGGGGCGAACGAGGCAGGCCCGTCTTCTTCGCGG

AAGCGTAAAAGGTGGGATGATGATTCGGGGTCCGAAGATGATGATTCGGGGGGCCCCGGGGAATCTCCAG

ATCCAGTGGAAATCGTTTACGAAGGGGATGCACAAGGGTACAACTGGGAGGGGAATTAGGTTGGCCGCCA

ACTTCGCCTGCCTTTCTATCTGAGTTCTTCCCTCTTGATGCTTTCGAACGACTCCTAAATTTCACAAAAT

CCTTTTTTTCTTATTTGAAATCCAAATCGAAATGCCTCAACTTGATAAATTAACTTATTTCTCACAATTC

TTCTGGTTATGTCTTCTCCTCTTTACTTTTTATATTCTCTTATTTAATAATAATAATGGAATACTTGGAA

TTAGTAGAATTCTCAAACTACGGAACCAACTGCTTTCGCACCGGGGGGGCGAGATCCGGAGCAAGGACCC

TAAGAATCTGGAAGATATCTCGAGAAAAGGTTTTAGCACCGGTCTCTCATATATGTACTCCAGTTTATCC

GAAGTATCCCAATGGTGTAAGACCGTCGACTATTTGGGAAAAAGGAGGAAAATCACTCTGATCTCTGATT

TCGGAGAAATAAGTGGCTCACGAGGAATGGAGAGACAGATTCTCTATTTGATCTCGAAGTCCTCATATAA

CACTTCTTCCAGTCGGATCACTTGTTGGAAAAACATAATGCTCACACATGTTCCACACGGGCAAGGAAGC

ATAATATCATGAAAGCCGTCTGATAATCTTTCTATAGGTTCCCCGAAGAGAATGGAAAAATCACAAAAAC

ACTTTCTATATGTTCTGTTATTTCGAGATTCGAAGAAGCAAGCAAGTTTTTCATATAGCTGTTTCCTGTG

TAGTGATCTCTGTTCGTGGAGATCATGAGGCCCCTGATACCCTGTGGTACGGGGGGGGGTAGAAGAGTGG

GCATGTGGGCTTCTATCAAAAGAGGAAAAGATACCGCTATCGCTATGCTCATATCTCCCTTCCCTCCTCC

AGGTGAGAAGCTGGTGTTGAGAAATGACATCCAATCTTTTGGTATCGATCATATATTCTAGGTGGATCAC

TTTTTTTCTTCAACTGAAATGGGCTGATCTAGTGTTTTTAAATGACCTTCCCCGATCAGAGAAGGGCAAG

AACTCTCTTGAACAGTGAGGAGCACAACGATTTGTGCTCTGAGCGATACAGCTTCTGTAAAGGAGTACAA

GGTGCTGTGCTAATAAAAGGAGAAATGAGATAAGGAGCAGTAAGGAGGATAAAAGAAGTAGAGTTAGGTT

CTTGTGTGTAATCGATATGTCTCCCGGGAGAAAACCCTCTATCAACACAACTATCCGAACACCTATAGCT

AGCTTGCTTTAGCTCCAACT

Supplementary Figure-2 Aligned bread and durum wheat sequences for the gene *ccmfc* obtained from the GenBank Accession:

nad-1

```
GU985444.1:61950-62335        TTACTAGACCAACCTGCTATAATTATTCCATAAACACCTAGCGAAGATATGGCAAACAAA
KJ078650.1:226434-226819      TTACTAGACCAACCTGCTATAATTATTCCATAAACACCTAGCGAAGATATGGCAAACAAA
                              ************************************************************

GU985444.1:61950-62335        TAAAGTAGCCCTATGTTCGGATCTGACAATACCATACCATAATCAAAAGGTACAACGGCC
KJ078650.1:226434-226819      TAAAGTAGCCCTATGTTCGGATCTGACAATACCATACCATAATCAAAAGGTACAACGGCC
                              ************************************************************

GU985444.1:61950-62335        CAAGCGACCAGACTTAACATAAATGTAGCCACTGGAGCCATTCTAAAAAGGGAGAAATTA
KJ078650.1:226434-226819      CAAGCGACCAGACTTAACATAAATGTAGCCACTGGAGCCATTCTAAAAAGGGAGAAATTA
                              ************************************************************

GU985444.1:61950-62335        GCACTACTTGGTGAAATAGGTTCTTTTAGAATCAATTTCAAACCATCTGCTAGAGGTTGT
KJ078650.1:226434-226819      GCACTACTTGGTGAAATAGGTTCTTTTAGAATCAATTTCAAACCATCTGCTAGAGGTTGT
                              ************************************************************

GU985444.1:61950-62335        AACAATCCGAACGATCCCACTACATCAGGACCCTTTCGACGTTGCACAAAAGCCATTACT
KJ078650.1:226434-226819      AACAATCCGAACGATCCCACTACATCAGGACCCTTTCGACGTTGCACAAAAGCCATTACT
                              ************************************************************

GU985444.1:61950-62335        TTACGTTCAGCTAGCACTAAAAAGGCTACTCCTAGTAGAAGTGGTAGAATTAAACAAAGT
KJ078650.1:226434-226819      TTACGTTCAGCTAGCACTAAAAAGGCTACTCCTAGTAGAAGTGGTAGAATTAAACAAAGT
                              ************************************************************

GU985444.1:61950-62335        ATTTCCGCTGGAACAGCTATGTACGT
KJ078650.1:226434-226819      ATTTCCGCTGGAACAGCTATGTACGT
                              *************************
```

nad-3 gene

```
GU985444.1:181883-182239      ATGTCGGAATTTGCACCTATTTGTATCTATTTAGTGATCAGTCCGCTAGTTTCTTTGATT
KJ078650.1:343021-343377      ATGTCGGAATTTGCACCTATTTGTATCTATTTAGTGATCAGTCCGCTAGTTTCTTTGATT
                              ************************************************************

GU985444.1:181883-182239      CCACTCGGTGTTCCTTTTCCATTTGCTTCCAATAGTTCGACCTATCCAGAAAAATTGTCG
KJ078650.1:343021-343377      CCACTCGGTGTTCCTTTTCCATTTGCTTCCAATAGTTCGACCTATCCAGAAAAATTGTCG
                              ************************************************************

GU985444.1:181883-182239      GCCTACGAATGTGGTTCCGATCCCTCCGGTGATGCCAGAAGTCGTTTCGATATACGATTT
KJ078650.1:343021-343377      GCCTACGAATGTGGTTCCGATCCCTCCGGTGATGCCAGAAGTCGTTTCGATATACGATTT
                              ************************************************************

GU985444.1:181883-182239      TATCCGGTTCCTATTTTATTTATTATCCCTGATCCGGAAGTCACCTTTTCTTTTCCTTGG
KJ078650.1:343021-343377      TATCCGGTTCCTATTTTATTTATTATCCCTGATCCGGAAGTCACCTTTTCTTTTCCTTGG
                              ************************************************************

GU985444.1:181883-182239      GCAGTACCTCCTAACAAGATTGATCTGTTTGGATCTTGGTCCATGATGGCCTTTTTATTG
KJ078650.1:343021-343377      GCAGTACCTCCTAACAAGATTGATCTGTTTGGATCTTGGTCCATGATGGCCTTTTTATTG
                              ************************************************************

GU985444.1:181883-182239      ATTTTGACGATTGGATCTCTCTATGAATGGAAAAGGGGTGCTTCGGATCGGGAGTAA
KJ078650.1:343021-343377      ATTTTGACGATTGGATCTCTCTATGAATGGAAAAGGGGTGCTTCGGATCGGGAGTAA
                              ***********************************************************
```

nad5 gene

```
GU985444.1:323719-323949      ATGTATCTACTTATTGTCTTTTTGCCTTTGCTCGGTAGTTCCGTAGCCGGTTTTTTCGGA
KJ078650.1:376970-377200      ATGTATCTACTTATTGTCTTTTTGCCTTTGCTCGGTAGTTCCGTAGCCGGTTTTTTCGGA
                              ************************************************************

GU985444.1:323719-323949      CGTTTTCTAGGATCTGAAGGAACCGCTATAATGACCACCACGTGCGTTTCATTTTCTTCG
KJ078650.1:376970-377200      CGTTTTCTAGGATCTGAAGGAACCGCTATAATGACCACCACGTGCGTTTCATTTTCTTCG
                              ************************************************************

GU985444.1:323719-323949      ATCTTATCTTTGATTGCTTTTTATGAAGTCGCACTGGGAGCTAGTGCTTGCTATCTCAGA
KJ078650.1:376970-377200      ATCTTATCTTTGATTGCTTTTTATGAAGTCGCACTGGGAGCTAGTGCTTGCTATCTCAGA
                              ************************************************************

GU985444.1:323719-323949      ATAGCTCCATGGATCTCATCGGAAATGTTTGATGCTTCTTGGGGCTTCTTT
KJ078650.1:376970-377200      ATAGCTCCATGGATCTCATCGGAAATGTTTGATGCTTCTTGGGGCTTCTTT
                              ***************************************************
```

nad6 gene

```
GU985444.1:338493-339182      ATGCGTCTTCTTGCTCCAGCATTCAAGTTCCATTTCAAGGGAGGACGACGTACCATGATA
KJ078650.1:361733-362422      ATGCGTCTTCTTGCTCCAGCATTCAAGTTCCATTTCAAGGGAGGACGACGTACCATGATA
                              ************************************************************

GU985444.1:338493-339182      CTTTCTGTTTTGTCGAGCCCTGCTTTGGTCTCTGGTTTGATGGTTGTACGTGCTAAAAAT
KJ078650.1:361733-362422      CTTTCTGTTTTGTCGAGCCCTGCTTTGGTCTCTGGTTTGATGGTTGTACGTGCTAAAAAT
                              ************************************************************

GU985444.1:338493-339182      CCGGTACATTCCGTTTTGTTTCCCATCCTAGTCTTTTGCGACACTTCTGGTTTACTTATT
KJ078650.1:361733-362422      CCGGTACATTCCGTTTTGTTTCCCATCCTAGTCTTTTGCGACACTTCTGGTTTACTTATT
                              ************************************************************

GU985444.1:338493-339182      TTGTTAGGTCTCGACTTCTCCGCTATGATCTCCCCAGTAGTTCATATAGGAGCTATTGCC
KJ078650.1:361733-362422      TTGTTAGGTCTCGACTTCTCCGCTATGATCTCCCCAGTAGTTCATATAGGAGCTATTGCC
                              ************************************************************

GU985444.1:338493-339182      GTTTCATTCCTATTCGTGGTTATGATGTTCAATATTCAAATAGCGGAGATTCACGAAGAA
KJ078650.1:361733-362422      GTTTCATTCCTATTCGTGGTTATGATGTTCAATATTCAAATAGCGGAGATTCACGAAGAA
                              ************************************************************

GU985444.1:338493-339182      GTATTGCGCTATTTACCAGTGAGTGGTATTATTGGACTGATCTTTTGGTGGGAAATGTTC
KJ078650.1:361733-362422      GTATTGCGCTATTTACCAGTGAGTGGTATTATTGGACTGATCTTTTGGTGGGAAATGTTC
                              ************************************************************

GU985444.1:338493-339182      TTCATTTTAGATAATGAAACCATTCCATTACTACCAACCCACAGAAATACGACCTCTCTG
KJ078650.1:361733-362422      TTCATTTTAGATAATGAAACCATTCCATTACTACCAACCCACAGAAATACGACCTCTCTG
                              ************************************************************

GU985444.1:338493-339182      AGATATACGGTTTATGCCGGAAAGGTACGAAGTTGGACTAATTTGGAAACATTGGGCAAT
KJ078650.1:361733-362422      AGATATACGGTTTATGCCGGAAAGGTACGAAGTTGGACTAATTTGGAAACATTGGGCAAT
                              ************************************************************

GU985444.1:338493-339182      TTGCTTTATACCTACTATTCCGTCTGGTTTTTGGTTTCTAGTCTGATTTTATTAGTAGCT
KJ078650.1:361733-362422      TTGCTTTATACCTACTATTCCGTCTGGTTTTTGGTTTCTAGTCTGATTTTATTAGTAGCT
                              ************************************************************

GU985444.1:338493-339182      ATGATTGGGGCTATAGTACTTACTATGCATAGGACTACAAAGGTGAAAAGACAGGATGTA
KJ078650.1:361733-362422      ATGATTGGGGCTATAGTACTTACTATGCATAGGACTACAAAGGTGAAAAGACAGGATGTA
                              ************************************************************

GU985444.1:338493-339182      TTCCGACGAAATGCCTTGGATTCTAGGAGGAATATAATGAACAGGACTATTTCTCCTTTT
KJ078650.1:361733-362422      TTCCGACGAAATGCCTTGGATTCTAGGAGGAATATAATGAACAGGACTATTTCTCCTTTT
                              ************************************************************

GU985444.1:338493-339182      GGCCATAGCCATAGAAGAAGCTTCTCCTCC
KJ078650.1:361733-362422      GGCCATAGCCATAGAAGAAGCTTCTCCTCC
                              ******************************
```

Cytochrome Oxidase

cox-1 gene

```
GU985444.1:301219-302789      ATGACAAATATGGTTCGATGGCTCTTCTCTACTAACCACAAGGATATTGGGACTCTCTAT
KJ078650.1:265395-266965      ATGACAAATATGGTTCGATGGCTCTTCTCTACTAACCACAAGGATATTGGGACTCTCTAT
                              ************************************************************

GU985444.1:301219-302789      TTCATCTTCGGTGCCATTGCAGGAGTGATGGGCACATGCTTCTCCGTACTGATTCGTATG
KJ078650.1:265395-266965      TTCATCTTCGGTGCCATTGCAGGAGTGATGGGCACATGCTTCTCCGTACTGATTCGTATG
                              ************************************************************

GU985444.1:301219-302789      GAATTAGCCCGACCCGGCGATCAAATTCTTGGTGGGAATCATCAACTTTATAATGTTTTA
KJ078650.1:265395-266965      GAATTAGCCCGACCCGGCGATCAAATTCTTGGTGGGAATCATCAACTTTATAATGTTTTA
                              ************************************************************

GU985444.1:301219-302789      ATAACGGCTCATGCTTTTTTTAATGATCTTTTTTTATGGTTATGCCGGCGATGATAGGTGGA
KJ078650.1:265395-266965      ATAACGGCTCATGCTTTTTTTAATGATCTTTTTTTATGGTTATGCCGGCGATGATAGGTGGA
                              ************************************************************

GU985444.1:301219-302789      TTTGGTAATTGGTTTGTTCCGATTCTGATAGGTGCACCTGACATGGCATTTCCACGATTA
KJ078650.1:265395-266965      TTTGGTAATTGGTTTGTTCCGATTCTGATAGGTGCACCTGACATGGCATTTCCACGATTA
```

```
                                ************************************************************

GU985444.1:301219-302789        AATAATATATCATTCTGGTTGTTGCCACCAAGTCTCTTGCTCCTATTAAGCTCAGCCTTA
KJ078650.1:265395-266965        AATAATATATCATTCTGGTTGTTGCCACCAAGTCTCTTGCTCCTATTAAGCTCAGCCTTA
                                ************************************************************

GU985444.1:301219-302789        GTAGAAGTGGGCAGCGGCACTGGGTGGACAGTCTATCCGCCCTTAAGTGGTATTACCAGC
KJ078650.1:265395-266965        GTAGAAGTGGGCAGCGGCACTGGGTGGACAGTCTATCCGCCCTTAAGTGGTATTACCAGC
                                ************************************************************

GU985444.1:301219-302789        CATTCTGGAGGAGCAGTTGATTTAGCAATTTTTAGTCTTCATCTATCAGGTATTTCATCA
KJ078650.1:265395-266965        CATTCTGGAGGAGCAGTTGATTTAGCAATTTTTAGTCTTCATCTATCAGGTATTTCATCA
                                ************************************************************

GU985444.1:301219-302789        ATTTTAGGTTCTATCAATTTTATAACAACTATCTTCAACATGCGTGGACCTGGAATGACT
KJ078650.1:265395-266965        ATTTTAGGTTCTATCAATTTTATAACAACTATCTTCAACATGCGTGGACCTGGAATGACT
                                ************************************************************

GU985444.1:301219-302789        ATGCATAGATTACCACTTTTTTGTGTGGTCCGTTCTAGTGACAGCATTCCTACTTTTATTA
KJ078650.1:265395-266965        ATGCATAGATTACCACTTTTTTGTGTGGTCCGTTCTAGTGACAGCATTCCTACTTTTATTA
                                ************************************************************

GU985444.1:301219-302789        TCACTTCCGGTACTGGCGGGGGCAATTACAATGTTATTAACCGATCGAAACTTTAATACA
KJ078650.1:265395-266965        TCACTTCCGGTACTGGCGGGGGCAATTACAATGTTATTAACCGATCGAAACTTTAATACA
                                ************************************************************

GU985444.1:301219-302789        ACCTTTTTTGATCCTGCAGGAGGGGGAGACCCAATATTATACCAGCATCTCTTTTGGTTC
KJ078650.1:265395-266965        ACCTTTTTTGATCCTGCAGGAGGGGGAGACCCAATATTATACCAGCATCTCTTTTGGTTC
                                ************************************************************

GU985444.1:301219-302789        TTCGGTCATCCAGAGGTGTATATTCTCATTCTGCCTGGATTTGGTATTATTAGTCATATC
KJ078650.1:265395-266965        TTCGGTCATCCAGAGGTGTATATTCTCATTCTGCCTGGATTTGGTATTATTAGTCATATC
                                ************************************************************

GU985444.1:301219-302789        GTATCGACCTTTTCAAGAAAACCGGTCTTCGGGTATCTAGGCATGGTTTATGCCATGATA
KJ078650.1:265395-266965        GTATCGACCTTTTCAAGAAAACCGGTCTTCGGGTATCTAGGCATGGTTTATGCCATGATA
                                ************************************************************

GU985444.1:301219-302789        AGTATAGGTGTTCTTGGATTTCTAGTTTGGGCTCATCATATGTTTACTGTGGGCTTAGAC
KJ078650.1:265395-266965        AGTATAGGTGTTCTTGGATTTCTAGTTTGGGCTCATCATATGTTTACTGTGGGCTTAGAC
                                ************************************************************

GU985444.1:301219-302789        GTTGATACGCGTGCCTACTTCACCGCAGCTACCATGATCATAGCTGTGCCCACAGGAATC
KJ078650.1:265395-266965        GTTGATACGCGTGCCTACTTCACCGCAGCTACCATGATCATAGCTGTGCCCACAGGAATC
                                ************************************************************

GU985444.1:301219-302789        AAAATCTTTAGTTGGATCGCTACCATGTGGGGAGGTTCGATACAATACAAAACACCCATG
KJ078650.1:265395-266965        AAAATCTTTAGTTGGATCGCTACCATGTGGGGAGGTTCGATACAATACAAAACACCCATG
                                ************************************************************

GU985444.1:301219-302789        TTATTTGCTGTAGGGTTCATCTTTTTGTTCACCATAGGAGGGCTCACTGGAATAGTTCTA
KJ078650.1:265395-266965        TTATTTGCTGTAGGGTTCATCTTTTTGTTCACCATAGGAGGGCTCACTGGAATAGTTCTA
                                ************************************************************

GU985444.1:301219-302789        GCAAACTCTGGGCTAGACATTGCTCTACATGATACTTATTATGTGGTTGCACATTTCCAT
KJ078650.1:265395-266965        GCAAACTCTGGGCTAGACATTGCTCTACATGATACTTATTATGTGGTTGCACATTTCCAT
                                ************************************************************

GU985444.1:301219-302789        TATGTACTTTCTATGGGAGCCGTTTTTGCTTTATTTGCTGGATTTTACTATTGGGTGGGT
KJ078650.1:265395-266965        TATGTACTTTCTATGGGAGCCGTTTTTGCTTTATTTGCTGGATTTTACTATTGGGTGGGT
                                ************************************************************

GU985444.1:301219-302789        AAAATCTTTGGTCGGACATATCCTGAAACTTTAGGCCAAATCCATTTTTGGATCACTTTT
KJ078650.1:265395-266965        AAAATCTTTGGTCGGACATATCCTGAAACTTTAGGCCAAATCCATTTTTGGATCACTTTT
                                ************************************************************

GU985444.1:301219-302789        TTCGGGGTTAATCTGACCTTCTTTCCCATGCATTTCTTAGGGCTTTCGGGTATGCCACGT
KJ078650.1:265395-266965        TTCGGGGTTAATCTGACCTTCTTTCCCATGCATTTCTTAGGGCTTTCGGGTATGCCACGT
                                ************************************************************

GU985444.1:301219-302789        CGCATTCCAGATTATCCAGATGCTTACGCCGGATGGAATGCTCTGAGCAGTTTCGGTTCT
KJ078650.1:265395-266965        CGCATTCCAGATTATCCAGATGCTTACGCCGGATGGAATGCTCTGAGCAGTTTCGGTTCT
                                ************************************************************

GU985444.1:301219-302789        TATATATCCGTAGTTGGGATTCGTCGTTTCTTCGTAGTTGTCGCAATCACTTCAAGCAGT
KJ078650.1:265395-266965        TATATATCCGTAGTTGGGATTCGTCGTTTCTTCGTAGTTGTCGCAATCACTTCAAGCAGT
                                ************************************************************
```

```
GU985444.1:301219-302789      GGAAAGAACCAAAAAAGTGCGGAAAGTCCTTGGGCTGTTGAACAGAATCCAACCACACTA
KJ078650.1:265395-266965      GGAAAGAACCAAAAATGTGCGGAAAGTCCTTGGGCTGTTGAACAGAATCCAACCACACTA
                              *************** ******************************************

GU985444.1:301219-302789      GAATGGTTGGTACAAAGCCCTCCGGCCTTTCATACTTTTGGAGAACTTCCTGCGGTAAAA
KJ078650.1:265395-266965      GAATGGTTGGTACAAAGCCCTCCGGCCTTTCATACTTTTGGAGAACTTCCTGCGGTAAAA
                              ************************************************************

GU985444.1:301219-302789      GAGACAAAAAG
KJ078650.1:265395-266965      GAGACAAAAAG
                              ***********
```

Cox-2

```
GU985444.1:543217-543606      ATGATTCTTCGTTCATTATCATGTCGATTCCTCACAATCGCTCTTTGTGATGCTGCGGAA
KJ078650.1:301995-302384      ATGATTCTTCGTTCATTATCATGTCGATTCCTCACAATCGCTCTTTGTGATGCTGCGGAA
                              ************************************************************

GU985444.1:543217-543606      CCATGGCAATTAGGATCTCAAGACGCAGCAACACCTATGATGCAAGGAATCATTGACTTA
KJ078650.1:301995-302384      CCATGGCAATTAGGATCTCAAGACGCAGCAACACCTATGATGCAAGGAATCATTGACTTA
                              ************************************************************

GU985444.1:543217-543606      CATCACGATATCTTTTTCTTCCTCATTCTTATTTTGGTTTTCGTATCACGGATGTTGGTT
KJ078650.1:301995-302384      CATCACGATATCTTTTTCTTCCTCATTCTTATTTTGGTTTTCGTATCACGGATGTTGGTT
                              ************************************************************

GU985444.1:543217-543606      CGCGCTTTATGGCATTTCAACGAGCAAACTAATCCAATCCCACAAAGGATTGTTCATGGA
KJ078650.1:301995-302384      CGCGCTTTATGGCATTTCAACGAGCAAACTAATCCAATCCCACAAAGGATTGTTCATGGA
                              ************************************************************

GU985444.1:543217-543606      ACTACTATCGAAATTATTCGGACCATATTTCCAAGTGTCATTCTTTTGTTCATTGCTATA
KJ078650.1:301995-302384      ACTACTATCGAAATTATTCGGACCATATTTCCAAGTGTCATTCTTTTGTTCATTGCTATA
                              ************************************************************


GU985444.1:543217-543606      CCATCGTTTGCTCTGTTATACTCAATGGACGGGGTATTAGTAGATCCAGCCATTACTATC
KJ078650.1:301995-302384      CCATCGTTTGCTCTGTTATACTCAATGGACGGGGTATTAGTAGATCCAGCCATTACTATC
                              ************************************************************

GU985444.1:543217-543606      AAAGCTATTGGACATCAATGGTATCGGAGT
KJ078650.1:301995-302384      AAAGCTATTGGACATCAATGGTATCGGAGT
                              ******************************

GU985444.1:543217-543606      ATGATTCTTCGTTCATTATCATGTCGATTCCTCACAATCGCTCTTTGTGATGCTGCGGAA
KJ078650.1:301995-302384      ATGATTCTTCGTTCATTATCATGTCGATTCCTCACAATCGCTCTTTGTGATGCTGCGGAA
                              ************************************************************

GU985444.1:543217-543606      CCATGGCAATTAGGATCTCAAGACGCAGCAACACCTATGATGCAAGGAATCATTGACTTA
KJ078650.1:301995-302384      CCATGGCAATTAGGATCTCAAGACGCAGCAACACCTATGATGCAAGGAATCATTGACTTA
                              ************************************************************

GU985444.1:543217-543606      CATCACGATATCTTTTTCTTCCTCATTCTTATTTTGGTTTTCGTATCACGGATGTTGGTT
KJ078650.1:301995-302384      CATCACGATATCTTTTTCTTCCTCATTCTTATTTTGGTTTTCGTATCACGGATGTTGGTT
                              ************************************************************

GU985444.1:543217-543606      CGCGCTTTATGGCATTTCAACGAGCAAACTAATCCAATCCCACAAAGGATTGTTCATGGA
KJ078650.1:301995-302384      CGCGCTTTATGGCATTTCAACGAGCAAACTAATCCAATCCCACAAAGGATTGTTCATGGA
                              ************************************************************

GU985444.1:543217-543606      ACTACTATCGAAATTATTCGGACCATATTTCCAAGTGTCATTCTTTTGTTCATTGCTATA
KJ078650.1:301995-302384      ACTACTATCGAAATTATTCGGACCATATTTCCAAGTGTCATTCTTTTGTTCATTGCTATA
                              ************************************************************

GU985444.1:543217-543606      CCATCGTTTGCTCTGTTATACTCAATGGACGGGGTATTAGTAGATCCAGCCATTACTATC
KJ078650.1:301995-302384      CCATCGTTTGCTCTGTTATACTCAATGGACGGGGTATTAGTAGATCCAGCCATTACTATC
                              ************************************************************

GU985444.1:543217-543606      AAAGCTATTGGACATCAATGGTATCGGAGT
KJ078650.1:301995-302384      AAAGCTATTGGACATCAATGGTATCGGAGT
                              ******************************
```

cox 3 gene

```
GU985444.1:85376-86173      ATGATTGAATCTCAGAGGCATTCTTATCATTTGGTAGATCCAAGTCCATGGCCTATTTCG
KJ078650.1:70264-71061      ATGATTGAATCTCAGAGGCATTCTTATCATTTGGTAGATCCAAGTCCATGGCCTATTTCG
                            ************************************************************

GU985444.1:85376-86173      GGTTCACTCGGAGCTTTGGCAACCACCGTAGGAGGTGTGATGTACATGCACTCATTTCAA
KJ078650.1:70264-71061      GGTTCACTCGGAGCTTTGGCAACCACCGTAGGAGGTGTGATGTACATGCACTCATTTCAA
                            ************************************************************

GU985444.1:85376-86173      GGGGGTGCAACACTTCTCAGTTTGGGCCTAATATTTCTCCTTTATACCATGTTCGTATGG
KJ078650.1:70264-71061      GGGGGTGCAACACTTCTCAGTTTGGGCCTAATATTTCTCCTTTATACCATGTTCGTATGG
                            ************************************************************

GU985444.1:85376-86173      TGGCGGGATGTTCTACGTGAATCCACGTTGGAAGGGCATCATACAAAAGCTGTACAATTA
KJ078650.1:70264-71061      TGGCGGGATGTTCTACGTGAATCCACGTTGGAAGGGCATCATACAAAAGCTGTACAATTA
                            ************************************************************

GU985444.1:85376-86173      GGACCTCGATATGGTTCTATTCTCTTCATAGTCTCGGAGGTTATGTTCCTTTTTGCTTTT
KJ078650.1:70264-71061      GGACCTCGATATGGTTCTATTCTCTTCATAGTCTCGGAGGTTATGTTCCTTTTTGCTTTT
                            ************************************************************

GU985444.1:85376-86173      TTTTGGGCTTCTTCTCATTCTTCTTTGGCACCTACGGTAGAGATCGGAGGTATTTGGCCC
KJ078650.1:70264-71061      TTTTGGGCTTCTTCTCATTCTTCTTTGGCACCTACGGTAGAGATCGGAGGTATTTGGCCC
                            ************************************************************

GU985444.1:85376-86173      CCAAAAGGGATTGGGGTTTTAGATCCTTGGGAAATCCCTCTTCTTAATACCCCTATTCTC
KJ078650.1:70264-71061      CCAAAAGGGATTGGGGTTTTAGATCCTTGGGAAATCCCTCTTCTTAATACCCCTATTCTC
                            ************************************************************

GU985444.1:85376-86173      CCTTCATCCGGAGCTGCCGTAACTTGGGCTCATCATGCTATACTCGCGGGGAAGGAAAAA
KJ078650.1:70264-71061      CCTTCATCCGGAGCTGCCGTAACTTGGGCTCATCATGCTATACTCGCGGGGAAGGAAAAA
                            ************************************************************

GU985444.1:85376-86173      CGAGCAGTTTACGCTTTAGTAGCAACCGTTTCACTGGCTCTAGTATCCACTGGCTTTCAA
KJ078650.1:70264-71061      CGAGCAGTTTACGCTTTAGTAGCAACCGTTTCACTGGCTCTAGTATCCACTGGCTTTCAA
                            ************************************************************


GU985444.1:85376-86173      GGAATGGAATATTACCAAGCACCCTCCACTATTTCGGATAGTATTTATGGTTCTACCTTT
KJ078650.1:70264-71061      GGAATGGAATATTACCAAGCACCCTCCACTATTTCGGATAGTATTTATGGTTCTACCTTT
                            ************************************************************

GU985444.1:85376-86173      TTCTTAGCAACTGGCTTTCATGGTTTTCATGTGATTATAGGTACTCTTTTCTTGATCGTA
KJ078650.1:70264-71061      TTCTTAGCAACTGGCTTTCATGGTTTTCATGTGATTATAGGTACTCTTTTCTTGATCGTA
                            ************************************************************

GU985444.1:85376-86173      TGTGGTATTCGCCAATATCTTGGTCATCTGACCAAGAAGCATCACGTTGGCTTTGAAGCA
KJ078650.1:70264-71061      TGTGGTATTCGCCAATATCTTGGTCAGATGACCAAGAAGCATCACGTTGGCTTTGAAGCA
                            **************************  ********************************

GU985444.1:85376-86173      GCTGCATGGTACTGGCATTTTGTAGACGTGGTTCGGTTATTCCCATTTGTCTCTATCTAT
KJ078650.1:70264-71061      GCTGCATGGTACTGGCATTTTGTAGACGTGGTTCGGTTATTCCCATTTGTCTCTATCTAT
                            ************************************************************

GU985444.1:85376-86173      TGGTGGGGAGGTATATGA
KJ078650.1:70264-71061      TGGTGGGGAGGTATATGA
                            ******************
```

CCMFC Cytochrome maturation gene

```
GU985444.1:22053-24373        ATGGTCCAACTACAGAACTTCTTCTTTTTCATTACTTCCATGGTCGTGCCCCGTGGCACG
KJ078650.1:129160-131479      ATGGTCCAACTACAGAACTTCTTCTTTTTCATTACTTCCATGGTCGTGCCCCGTGGCACG
                              ************************************************************

GU985444.1:22053-24373        GCAGCACCCGTACTATTGAAATGGTTCGTCAGTAGAGATGTTCCCACTGGTGCCTCTTCT
KJ078650.1:129160-131479      GCAGCACCCGTACTATTGAAATGGTTCGTCAGTAGAGATGTTCCCACTGGTGCCTCTTCT
                              ************************************************************

GU985444.1:22053-24373        TCCAATGGTACTATAATTCCTATTCCTATCCCTTTATTCCCTTTTTTGGTCTATCTACAT
KJ078650.1:129160-131479      TCCAATGGTACTATAATTCCTATTCCTATCCCTTTATTCCCTTTTTTGGTCTATCTACAT
```

```
                                 ************************************************************

GU985444.1:22053-24373           TTAAGGAAATTCATACGCTCCATGGACAGAGCAAAAAGTGGAGTGTTGGTCAAAGCAAGC
KJ078650.1:129160-131479         TTAAGGAAATTCATACGCTCCATGGACAGAGCAAAAAGTGGAGTGTTGGTCAAAGCAAGC
                                 ************************************************************

GU985444.1:22053-24373           CGCCCTATTCTATTACCAGACAAAATGGAGAGAAGCTCATCCGCTAGAAATGCTTTATTT
KJ078650.1:129160-131479         CGCCCTATTCTATTACCAGACAAAATGGAGAGAAGCTCATCCGCTAGAAATGCTTTATTT
                                 ************************************************************

GU985444.1:22053-24373           CGTTTCGTTCCCGTTCTTCATTTCCTTATTATCGAATCCATGGGGGACTTGTCATATTTA
KJ078650.1:129160-131479         CGTTTCGTTCCCGTTCTTCATTTCCTTATTATCGAATCCATGGGGGACTTGTCATATTTA
                                 ************************************************************

GU985444.1:22053-24373           GAATCTTTCTGCGGTCTGCTCTGTTTACAATTCTTTCGTACTCTCTTCTCTTTACCACGC
KJ078650.1:129160-131479         GAATCTTTCTGCGGTCTGCTCTGTTTACAATTCTTTCGTACTCTCTTCTCTTTACCACGC
                                 ************************************************************

GU985444.1:22053-24373           GATAGGTCAGCGAAGCGTGAGCGGGCGCTCCGAAGTAAAGGCCAAACACTTCGGCCTAAG
KJ078650.1:129160-131479         GATAGGTCAGCGAAGCGTGAGCGGGCGCTCCGAAGTAAAGGCCAAACACTTCGGCCTAAG
                                 ************************************************************

GU985444.1:22053-24373           GGGAATGAGCAACAAAATGACAAGATGAGGTGCCCCGGGCACCCCCATATAGAAAGAAGG
KJ078650.1:129160-131479         GGGAATGAGCAACAAAATGACAAGATGAGGTGCCCCGGGCACCCCCATATAGAAAGAAGG
                                 ************************************************************

GU985444.1:22053-24373           GTCGAAGGTTTTGGGCCTGTAGCTTTCCCCGCCCCCCCCTCGTCGAGTGGTGCTTGTTTG
KJ078650.1:129160-131479         GTCGAAGGTTTTGGGCCTGTAGCTTTCCCCGCCCCCCCCTCGTCGAGTGGTGCTTGTTTG
                                 ************************************************************

GU985444.1:22053-24373           GGGGGTGTGCCACCAGAAAGCGGGCTTGAAGCTCTCGCCTTACCAACGAGCCGACTGCTG
KJ078650.1:129160-131479         GGGGGTGTGCCACCAGAAAGCGGGCTTGAAGCTCTCGCCTTACCAACGAGCCGACTGCTG
                                 ************************************************************

GU985444.1:22053-24373           ATGGCTGTTGGTCACGACTACTACAAAAAAGTGAAGATGAATCTTTCTATTTCACATGGA
KJ078650.1:129160-131479         ATGGCTGTTGGTCACGACTACTACAAAAAAGTGAAGATGAATCTTTCTATTTCACATGGA
                                 ************************************************************

GU985444.1:22053-24373           GGAGTGTGCATCTTTATGTTGGGTGTTCTTCTGTCGTGCGACCCGATGGCTTATGTGCGA
KJ078650.1:129160-131479         GGAGTGTGCATCTTTATGTTGGGTGTTCTTCTGTCGTGCGACCCGATGGCTTATGTGCGA
                                 ************************************************************

GU985444.1:22053-24373           CCTGTGGCCCACGCCTCCTATTCTATTTGTTCAGGGCGGGCGGCGTGAACTCTGATTCGA
KJ078650.1:129160-131479         CCTGTGGCCCACGCCTCCTATTCTATTTGTTCAGGGCGGGCGGCGTGAACTCTGATTCGA
                                 ************************************************************

GU985444.1:22053-24373           TCCGGGTATTCAATCCCGCCGCTGAGATGCTCAGTTGACTCCTTAACCTTGATAGGAAGA
KJ078650.1:129160-131479         TCCGGGTATTCAATCCCGCCGCTGAGATGCTCAGTTGACTCCTTAACCTTGATAGGAAGA
                                 ************************************************************

GU985444.1:22053-24373           TGGCTTATTCCAAAATTCGTGCATAAGGGTAAGGAACTTTGGATGAATTAATGCGAATGG
KJ078650.1:129160-131479         TGGCTTATTCCAAAATTCGTGCATAAGGGTAAGGAACTTTGGATGAATTAATGCGAATGG
                                 ************************************************************

GU985444.1:22053-24373           GTGTAAGCCTCGCTGCTCGGAAACACCCAGTGCTGACCACACTGAGAGACACGAAAGCGC
KJ078650.1:129160-131479         GTGTAAGCCTCGCTGCTCGGAAACACCCAGTGCTGACCACACTGAGAGACACGAAAGCGC
                                 ************************************************************

GU985444.1:22053-24373           AGGTAATGCCAGTTGGCGAAGTGGCGTTAAGCATCCCTAGCGGTACGCAAAGAGAGGTCG
KJ078650.1:129160-131479         AGGTAATGCCAGTTGGCGAAGTGGCGTTAAGCATCCCTAGCGGTACGCAAAGAGAGGTCG
                                 ************************************************************

GU985444.1:22053-24373           TGATGATATCATCTACGTCCGTACCGCTCCTCGTGGAGTAGATCCCGCATCCAACCAACC
KJ078650.1:129160-131479         TGATGATATCATCTACGTCCGTACCGCTCCTCGTGGAGTAGATCCCGCATCCAACCAACC
                                 ************************************************************

GU985444.1:22053-24373           CTTTTTTGACCAGGGAACGGGAGAATTCCCACTACCGCTGGCAGGCCAGCCGGGCCGTGA
KJ078650.1:129160-131479         CTTTTTTGACCAGGGAACGGGAGAATTCCCACTACCGCTGGCAGGCCAGCCGGGCCGTGA
                                 ************************************************************


GU985444.1:22053-24373           GCGCGGTGGGAACGGGCTTCCCAAAAAGCGAGCCCCGGCCCGGGTCAGCATAGAATGGGG
KJ078650.1:129160-131479         GCGCGGTGGGAACGGGCTTCCCAAAAAGCGAGCCCCGGCCCGGGTCAGCATAGAATGGGG
                                 ************************************************************

GU985444.1:22053-24373           GGGGACGGCCCTAATGTTGTGTTGGCCGGGTTGCGGGCGGATAAAAGCGGACGTGGGGAC
KJ078650.1:129160-131479         -GGGACGGCCCTAATGTTGTGTTGGCCGGGTTGCGGGCGGATAAAAGCGGACGTGGGGAC
                                  ************************************************************
```

```
GU985444.1:22053-24373        TCGGGTCGGGGCACAGCGTAACTAAGAGAGCCATTCCATTTAGGGCGAGACAGAATGGGC
KJ078650.1:129160-131479      TCGGGTCGGGGCACAGCGTAACTAAGAGAGCCATTCCATTTAGGGCGAGACAGAATGGGC
                              ************************************************************

GU985444.1:22053-24373        GGGCACAAGTGGTCTGGTGTCCGAGCCGATTGGTCAGACGACGACTACTGCACATATTAT
KJ078650.1:129160-131479      GGGCACAAGTGGTCTGGTGTCCGAGCCGATTGGTCAGACGACGACTACTGCACATATTAT
                              ************************************************************

GU985444.1:22053-24373        ATTAGCCGCCTATCCCGGAATGGACTGGGATGGAATAGAAAGAACGCGAAGCAGCAAGCA
KJ078650.1:129160-131479      ATTAGCCGCCTATCCCGGAATGGACTGGGATGGAATAGAAAGAACGCGAAGCAGCAAGCA
                              ************************************************************

GU985444.1:22053-24373        AGGGATGAGCGCTTTGTTGCTAAAGCGCATACGTTTTCTTGCTGGGTCGGTTTGTTTTTG
KJ078650.1:129160-131479      AGGGATGAGCGCTTTGTTGCGCCTGCGCATACGTTTTCTTGCTGGGTCGGTTTGTTTTTG
                              ********************  ***********************************

GU985444.1:22053-24373        GGGGGTGGGGCAATAAGCTTGCTTCTTCACAAGCTTATCCCCGCCCCCTTTCCTGTCCGT
KJ078650.1:129160-131479      GGGGGTGGGGCAATAAGCTTGCTTCTTCACAAGCTTATCCCCGCCCCCTTTCCTGTCCGT
                              ************************************************************

GU985444.1:22053-24373        CCCGACCGGCAGCAGTTGGGTCTCCCCATCTCTCCTCAACTTCCCCGGTCTTCGGCCCGA
KJ078650.1:129160-131479      CCCGACCGGCAGCAGTTGGGTCTCCCCATCTCTCCTCAACTTCCCCGGTCTTCGGCCCGA
                              ************************************************************

GU985444.1:22053-24373        GCTGTATGAGGCAGAAACTCGTCCCACGTACGGTTCGGAGGCCGAGCCCCACCCCTGCAA
KJ078650.1:129160-131479      GCTGTATGAGGCAGAAACTCGTCCCACGTACGGTTCGGAGGCCGAGCCCCACCCCTGCAA
                              ************************************************************

GU985444.1:22053-24373        TAATGGTGCGGCTTAGGTCAACTAATACGAATAAGATACAGTTCACTCAACGATTGCCCT
KJ078650.1:129160-131479      TAATGGTGCGGCTTAGGTCAACTAATACGAATAAGATACAGTTCACTCAACGATTGCCCT
                              ************************************************************

GU985444.1:22053-24373        TGGGTCCCGAACTCCATATGGGGAAGGAACGTTGTTGTTTGCGAGGTCTCGATCATTTAC
KJ078650.1:129160-131479      TGGGTCCCGAACTCCATATGGGGAAGGAACGTTGTTGTTTGCGAGGTCTCGATCATTTAC
                              ************************************************************


GU985444.1:22053-24373        ATGGACCCACTTCTCATTCCATTTGTGGGAATTTGATGATTTATAAACCGTCCCCAACGA
KJ078650.1:129160-131479      ATGGACCCACTTCTCATTCCATTTGTGGGAATTTGATGATTTATAAACCGTCCCCAACGA
                              ************************************************************

GU985444.1:22053-24373        GCGAAAGGTTCATGTTTGAACATGATGAATCACTTCGTGCCGACCTGTTGCCCATAAACT
KJ078650.1:129160-131479      GCGAAAGGTTCATGTTTGAACATGATGAATCACTTCGTGCCGACCTGTTGCCCATAAACT
                              ************************************************************

GU985444.1:22053-24373        TTCCTGCCTCATATGAGAATGGAAAACTGGAAGATTTTCTGCATCGGTGGATGAAGAATC
KJ078650.1:129160-131479      TTCCTGCCTCATATGAGAATGGAAAACTGGAAGATTTTCTGCATCGGTGGATGAAGAATC
                              ************************************************************

GU985444.1:22053-24373        ACGAACATAAGAATTTCTGGTTTAGCATGTTCCCAGAAAGAAGATACTTTTTTTCCATTC
KJ078650.1:129160-131479      ACGAACATAAGAATTTCTGGTTTAGCATGTTCCCAGAAAGAAGATACTTTTTTTCCATTC
                              ************************************************************

GU985444.1:22053-24373        GAGAAACGAGGAGCACGACTGAAGTGGCTATACATACAAATCCATTTACGGATCTATATG
KJ078650.1:129160-131479      GAGAAACGAGGAGCACGACTGAAGTGGCTATACATACAAATCCATTTACGGATCTATATG
                              ************************************************************

GU985444.1:22053-24373        CTCCGATTGGAACTGGAAGTTCCAGAACTGGCGGCTGGTATACCACCATAATGAAATTGC
KJ078650.1:129160-131479      CTCCGATTGGAACTGGAAGTTCCAGAACTGGCGGCTGGTATACCACCATAATGAAATTGC
                              ************************************************************

GU985444.1:22053-24373        CTTTTATTTTTAGTATTCGGATAGGATTTCTGTTGGCTTCATCGGGAGGCTCGCGTAGTT
KJ078650.1:129160-131479      CTTTTATTTTTAGTATTCGGATAGGATTTCTGTTGGCTTCATCGGGAGGCTCGCGTAGTT
                              ************************************************************

GU985444.1:22053-24373        TGTTACGTCAACTCCAAAAGGATAAGTTGCATTGGAATCGA
KJ078650.1:129160-131479      TGTTACGTCAACTCCAAAAGGATAAGTTGCATTGGAATCGA
                              *****************************************
```

CCMFN cytochrome maturation gene

```
GU985444.1:213152-213672      ATGTTCTTGGTGGATGCAGGCCCTGGTACCCCCAAAATTTGTATGCAAGATGAGCTTACA
KJ078650.1:10793-11313        ATGTTCTTGGTGGATGCAGGCCCTGGTACCCCAAAATTTGTATGCAAGATGAGCTTACA
                              ********************************* ***************************
```

```
GU985444.1:213152-213672      GGACTGCCAATCAAGCGAGCCACCAGGTTTGAGAATAAGGTGGGATCCAAGAATGTAGTG
KJ078650.1:10793-11313        GGACTGCCAATCAAGCGAGCCACCAGGTTTGAGAATAAGGTGGGATCCAAGAATGTAGTG
                              ************************************************************

GU985444.1:213152-213672      GCTGGTGAATCACTGATCAAAAAGCGGATTTTTGAGAGATTCTTCATCGATCTAGTGGCC
KJ078650.1:10793-11313        GCTGGTGAATCACTGATCAAAAAGCGGATTTTTGAGAGATTCTTCATCGATCTAGTGGCC
                              ************************************************************

GU985444.1:213152-213672      GGTGAATCACTGATCAAAGAGCGAGCAGCCGCCAGGTTTAATGATTTTGTGGGATCCCTG
KJ078650.1:10793-11313        GGTGAATCACTGATCAAAGAGCGAGCAGCCGCCAGGTTTAATGATTTTGTGGGATCCCTG
                              ************************************************************

GU985444.1:213152-213672      GATGTAGCGGCTGGCGAACCGCTTCTTCTTCCACAAAGATTCAGACAAAACCGAGCTTGG
KJ078650.1:10793-11313        GATGTAGCGGCTGGCGAACCGCTTCTTCTTCCACAAAGATTCAGACAAAACCGAGCTTGG
                              ************************************************************

GU985444.1:213152-213672      ATAGAACTGAAGAAGATTTGGCGAACGAAGAAAAAGGTCAAAGGGTTTATTATAAAAAAA
KJ078650.1:10793-11313        ATAGAACTGAAGAAGATTTGGCGAACGAAGAAAAAGGTCAAAGGGTTTATTATAAAAAAA
                              ************************************************************

GU985444.1:213152-213672      ATCAAAGGAGGTTATTCAGTAGCCATCGCGGGTTTCATTACTTTTCTTCCATTCAAAAAA
KJ078650.1:10793-11313        ATCAAAGGAGGTTATTCAGTAGCCATCGCGGGTTTCATTACTTTTCTTCCATTCAAAAAA
                              ************************************************************

GU985444.1:213152-213672      GCTCTTCTAAAAAAAAGGATAGCGAATGATCGATTCACCATTGATAGCATTAACCCTAAA
KJ078650.1:10793-11313        GCTCTTCTAAAAAAAAGGATAGCGAATGATCGATTCACCATTGATAGCATTAACCCTAAA
                              ************************************************************

GU985444.1:213152-213672      AGGAGGGATATTGTGATAATAGCGGCAGATCAAACAAGAAC
KJ078650.1:10793-11313        AGGAGGGATATTGTGATAATAGCGGCAGATCAAACAAGAAC
                              *****************************************
```

Open reading frames

Orf-173 *gene*

```
GU985444.1:561548-562069      TTAGGTGTAATAGGACTCCCAGTTACTGCGCGCGATCGTATACTGAGGTGCTCCCCGTCG
KJ078650.1:320333-320854      TTAGGTGTAATAGGACTCCCAGTTACTGCGCGCGATCGTATACTGAGGTGCTCCCCGTCG
                              ************************************************************

GU985444.1:561548-562069      GTTGTTGGAACGACGCGAGCCGGGCCGGGCCTCGATTCCTTTCAAAAAGATGAAGGGACA
KJ078650.1:320333-320854      GTTGTTGGAACGACGCGAGCCGGGCCGGGCCTCGATTCCTTTCAAAAAGATGAAGGGACA
                              ************************************************************

GU985444.1:561548-562069      GAGGTGACTAATTCCCCATCTCATTTGGGGCGGAAAACGAATCGACATCTCGATGTGATA
KJ078650.1:320333-320854      GAGGTGACTAATTCCCCATCTCATTTGGGGCGGAAAACGAATCGACATCTCGATGTGATA
                              ************************************************************

GU985444.1:561548-562069      CAGCCCTTTCCATTTTCGTTGGGAAAGAACGGCGAAGTCCATCCGAACCCTCCAATGAAG
KJ078650.1:320333-320854      CAGCCCTTTCCATTTTCGTTGGGAAAGAACGGCGAAGTCCATCCGAACCCTCCAATGAAG
                              ************************************************************

GU985444.1:561548-562069      AAATAAGAGGAGAGCAAAGCGCCAATGGCGCGCGAAGCGCATGCGGAAGGGGCACGGAGA
KJ078650.1:320333-320854      AAATAAGAGGAGAGCAAAGCGCCAATGGCGCGCGAAGCGCATGCGGAAGGGGCACGGAGA
                              ************************************************************

GU985444.1:561548-562069      AATAAAGAAGTGTGGGGGAGAAGCAGCCGAGCTCATTCCCTTCGCTTCCTGGGCCCAAAG
KJ078650.1:320333-320854      AATAAAGAAGTGTGGGGGAGAAGCAGCCGAGCTCATTCCCTTCGCTTCCTGGGCCCAAAG
                              ************************************************************

GU985444.1:561548-562069      CAGTGCTTTGTTTCCTGGCCAAATCAAGGATTTGGGGCTGATTGCAAAAGATATCTGAAT
KJ078650.1:320333-320854      CAGTGCTTTGTTTCCTGGCCAAATCAAGGATTTGGGGCTGATTGCAAAAGATATCTGAAT
                              ************************************************************

GU985444.1:561548-562069      AGAAAGATAGATCCATCCATCTATCCAGATATCTAAAAAAGAATCGATTTCGATTTCATG
KJ078650.1:320333-320854      AGAAAGATAGATCCATCCATCTATCCAGATATCTAAAAAAGAATCGATTTCGATTTCATG
                              ************************************************************

GU985444.1:561548-562069      AAACCTTTGATTCAAAGAACTGCGCTTAGCCCCCCCGCTCAT
KJ078650.1:320333-320854      AAACCTTTGATTCAAAGAACTGCGCTTAGCCCCCCCGCTCAT
                              *******************************************
```

Orf-240-genes

```
GU985444.1:70866-71588      ATGTCAGTTTCGTTATTACAACCTTATTTTTTTATGTCAAAGACAAAAAGCTACGCGCAA
KJ078650.1:84870-85592      ATGTCAGTTTCGTTATTACAACCTTATTTTTTTATGTCAAAGACAAGAAGCTACGCGCAA
                            ********************************************* ************

GU985444.1:70866-71588      ATTCTCATTGGATCTCGGTTGTTCTTAACAGCGATGGCTATTCATTTAAGTCTTCGGGTA
KJ078650.1:84870-85592      ATTCTCATTGGATCTCGGTTGTTCTTAACAGCGATGGCTATTCATTTAAGTCTTCGGGTA
                            ************************************************************

GU985444.1:70866-71588      GCACCACCAGATCTTCAACAAGGTGGAAATTCTCGTATTTCGTATGTACATGTTCCTGCG
KJ078650.1:84870-85592      GCACCACCAGATCTTCAACAAGGTGGAAATTCTCGTATTTCGTATGTACATGTTCCTGCG
                            ************************************************************

GU985444.1:70866-71588      GCTCGGATGAGTATAGTTATTTATATCGCGACAGCTATAAACAGTTCCTTGTTCCCATTA
KJ078650.1:84870-85592      GCTCGGATGAGTATAGTTATTTATATCGCGACAGCTATAAACAGTTCCTTGTTCCCATTA
                            ************************************************************

GU985444.1:70866-71588      ACAAAACATCCCCTTTTTCTTCGCTCTTCCGGAACCGGTACAGAAATTGGTGCTTTTTCT
KJ078650.1:84870-85592      ACAAAACATCCCCTTTTTCTTCGCTCTTCCGGAACCGGTACAGAAATTGGTGCTTTTTCT
                            ************************************************************

GU985444.1:70866-71588      ACTTTGTTTACGTTAGTGACTGGGGGGTTTCGGGGAAGGCCTATGTGGGGTACCTTTCGG
KJ078650.1:84870-85592      ACTTTGTTTACGTTAGTGACTGGGGGGTTTCGGGGAAGGCCTATGTGGGGTACCTTTCGG
                            ************************************************************

GU985444.1:70866-71588      GTGTGGGATGCTCGTTTAACTTCTGTATTCATCTTGTTCCTTATTTACCTGGGTGCACTG
KJ078650.1:84870-85592      GTGTGGGATGCTCGTTTAACTTCTGTATTCATCTTGTTCCTTATTTACCTGGGTGCACTG
                            ************************************************************

GU985444.1:70866-71588      CGTTTTCAAAAGCTTCCTGTCGAACCGGCTCCTATTTCAATCCGTGCTGGACCGATCGAT
KJ078650.1:84870-85592      CGTTTTCAAAAGCTTCCTGTCGAACCGGCTCCTATTTCAATCCGTGCTGGACCGATCGAT
                            ************************************************************

GU985444.1:70866-71588      ATACCAATAATAAAGTCTCCAGTCAACTGGTGGAATACATCGCATCAACCTGGGAGCATT
KJ078650.1:84870-85592      ATACCAATAATAAAGTCTCCAGTCAACTGGTGGAATACATCGCATCAACCTGGGAGCATT
                            ************************************************************

GU985444.1:70866-71588      AGCCGATCTGGTACATCAATACATGTTCCTATGCCCATTCCAATCTTGTCTAACTTTGCT
KJ078650.1:84870-85592      AGCCGATCTGGTACATCAATACATGTTCCTATGCCCATTCCAATCTTGTCTAACTTTGCT
                            ************************************************************

GU985444.1:70866-71588      AACTTCCCCTTCTCTACCCGTATCTTGTTCGTTCTGGAAACACGTCTTCCTATTCCATCT
KJ078650.1:84870-85592      AACTTCCCCTTCTCTACCCGTATCTTGTTCGTTCTGGAAACACGTCTTCCTATTCCATCT
                            ************************************************************

GU985444.1:70866-71588      TTTCCCGAATCTCCCTTAACGGAAGAAATAGAAGCTCGAGAAGGAATACCACTAAAAACC
KJ078650.1:84870-85592      TTTCCCGAATCTCCCTTAACGGAAGAAATAGAAGCTCGAGAAGGAATACCACTAAAAACC
                            ************************************************************

GU985444.1:70866-71588      TAG
KJ078650.1:84870-85592      TAG
                            ***
```

Orf-349 gene

```
GU985444.1:554801-555850      CTAGATATCCCCCGTGGGTTTGCATAAGTAACCTCCTATCAGATCCGATAATGTATCAGG
KJ078650.1:313579-314628      CTAGATATCCCCCGTGGGTTTGCATAAGTAACCTCCTATCAGATCCGATAATGTATCAGG
                              ************************************************************

GU985444.1:554801-555850      ATCAGACCCCCTCTCTGCTGGTTGCCAATCCAAGGGTTTGCAAAGCATCGGAAGATTGAG
KJ078650.1:313579-314628      ATCAGACCCCCTCTCTGCTGGTTGCCAATCCAAGGGTTTGCAAAGCATCGGAAGATTGAG
                              ************************************************************

GU985444.1:554801-555850      TTTGATAGGGAGCAGATTTATGTTCAAATTGCACATAACATAACAAGAATTCTCTATATA
KJ078650.1:313579-314628      TTTGATAGGGAGCAGATTTATGTTCAAATTGCACATAACATAACAAGAATTCTCTATATA
                              ************************************************************

GU985444.1:554801-555850      CCCTTGACCTTTCTTAAGAACCAGCACTGGATCTTCTTTTGTTACTCCCCTATCTGTTGA
KJ078650.1:313579-314628      CCCTTGACCTTTCTTAAGAACCAGCACTGGATCTTCTTTTGTTACTCCCCTATCTGTTGA
                              ************************************************************
```

```
GU985444.1:554801-555850      TATATGTAGAACATTTCTTTCAATCATGAATTGGAGCAAGTATTTCCCGATATCATAATG
KJ078650.1:313579-314628      TATATGTAGAACATTTCTTTCAATCATGAATTGGAGCAAGTATTTCCCGATATCATAATG
                              ************************************************************

GU985444.1:554801-555850      ACACTGCATTTTTTTCTTTTTCTCTTTTTTGCTAGCACCTTTGGGCTGAACAACATCCTT
KJ078650.1:313579-314628      ACACTGAATTTTTTTCTTTTTCTCTTTTTTGCTAGCACCTTTGGGCTGAACAACATCCTT
                              ****** ***********************************************************

GU985444.1:554801-555850      AACTGATTCTACCTTACTAGTGAGTACTGCTTCCACCTTACCACTACCTGGTGCTTTGTA
KJ078650.1:313579-314628      AACTGATTCTACCTTACTAGTGAGTACTGCTTCCACCTTACCACTACCTGGTGCTTTGTA
                              ************************************************************

GU985444.1:554801-555850      TTGTAGAAACCTTGCTTGTTCTCGTACAGTTGAATTAAGTTGATCTATAAATCTAGCTGC
KJ078650.1:313579-314628      TTGTAGAAACCTTGCTTGTTCTCGTACAGTTGAATTAAGTTGATCTATAAATCTAGCTGC
                              ************************************************************

GU985444.1:554801-555850      CTTAACTGCGGATGATTCCTGAAGAGTGTTGAATACCAAGCCTAGTACATGAATCATGAT
KJ078650.1:313579-314628      CTTAACTGCGGATGATTCCTGAAGAGTGTTGAATACCAAGCCTAGTACATGAATCATGAT
                              ************************************************************

GU985444.1:554801-555850      AGCCTCAAGCGTATAGATACCAAACATCTTAAGCATAGGTAAGCTACTTGGTAGGCAACC
KJ078650.1:313579-314628      AGCCTCAAGCGTATAGATACCAAACATCTTAAGCATAGGTAAGCTACTTGGTAGGCAACC
                              ************************************************************

GU985444.1:554801-555850      CTTTAGATATTGCTTAGCGTTAGGCTGATCTTTATTAATCAAGAACTTTACTATGTTAGG
KJ078650.1:313579-314628      CTTTAGATATTGCTTAGCGTTAGGCTGATCTTTATTAATCAAGAACTTTACTATGTTAGG
                              ************************************************************

GU985444.1:554801-555850      AGTTACTTTAAATAGATTATCCTCATCGAACTTCATTGTGCAATTTGCAATTTGATTCTG
KJ078650.1:313579-314628      AGTTACTTTAAATAGATTATCCTCATCGAACTTCATTGTGCAATTTGCAATTTGATTCTG
                              ************************************************************

GU985444.1:554801-555850      TAAATCTATTAATTCAGTATCGGAAAACACACCATTTTGATTCTTCTTTTCATTAATCAA
KJ078650.1:313579-314628      TAAATCTATTAATTCAGTATCGGAAAACACACCATTTTGATTCTTCTTTTCATTAATCAA
                              ************************************************************

GU985444.1:554801-555850      CAACTTTCTAACATCATCCTGATACATATCAACATTAGAAGTCTTTTTCGTTTTGTCAAT
KJ078650.1:313579-314628      CAACTTTCTAACATCATCCTGATACATATCAACATTAGAAGTCTTTTTCGTTTTGTCAAT
                              ************************************************************

GU985444.1:554801-555850      ATCCAACAGCTTAGCGTAGTAATCGTTCCAGAACTCCTTAATAATACGTGCATTTTGCAA
KJ078650.1:313579-314628      ATCCAACAGCTTAGCGTAGTAATCGTTCCAGAACTCCTTAATAATACGTGCATTTTGCAA
                              ************************************************************

GU985444.1:554801-555850      AGATTTCTCATTCTCAATGCTATCATCATATGTCTCGGAACAATAAAATAGAACCTGCAT
KJ078650.1:313579-314628      AGATTTCTCATTCTCAATGCTATCATCATATGTCTCGGAACAATAAAATAGAACCTGCAT
                              ************************************************************

GU985444.1:554801-555850      ACTTTTATTTGTTACCCTTACTAAACCGCAGTAAGGATACGAATACGACTGAAATAAACT
KJ078650.1:313579-314628      ACTTTTATTTGTTACCCTTACTAAACCGCAGTAAGGATACGAATACGACTGAAATAAACT
                              ************************************************************

GU985444.1:554801-555850      ACTAGAATCTAAATATAAGGAAAGGATCAG
KJ078650.1:313579-314628      ACTAGAATCTAAATATAAGGAAAGGATCAG
                              *******************************
```

Chapter 7

Conclusion and future directions

This Ph.D. study focussed on the hybridisation of hexaploid bread and tetraploid durum wheat for the transfer of desirable traits from one parent to another. The potential of inter-ploidy crosses between bread and durum wheat for improving a number of economically important traits has been outlined by a number of researchers (Kalous et al. 2015; Chapter 4; Chapter 5). Transferring chromosome segments from wild relatives or alien species into domesticated species is complicated (Friebe et al. 1996). Subsequent embryo rescue techniques may be needed after hybridisation for successful establishment of F_1 progenies. Even if successful F_1 progenies were established through subsequent rescue techniques, the hybrids may not survive to the next generation if they experience difficulties with uneven chromosome numbers and pairing. As an alternative, to overcome the complication that occurs through the alien introgression of wild or related species hybridisation, this study used domesticated bread and durum wheat species for inter-ploidy hybridisation (Chapter 2).

Domesticated bread and durum wheat belong to the same genus, *Triticum*, but they have different ploidy levels. The hybridisation between these two ploidy level wheat species may readily occur and F_1 seeds germinate without any subsequent rescue techniques (Martin et al. 2011; Eberhard et al. 2010). Most of the earliest work related to bread and durum wheat crosses was conducted by pioneering Japanese scientist Hitoshi Kihara who subsequently comprehensively reviewed over 50 years of research in the field (Kihara and Lilienfeld 1949; Kihara , 1925). Nevertheless for much of the subsequent 20th century hexaploid/tetraploid wheat crosses were not widely used in plant breeding programs for improving bread and durum wheat. The main reason why hexaploid/tetraploid cross failed to attract plant breeders attention is the incompatibility of the different ploidy species, Another major reason is the lack of efficient and high throughput techniques to characterise the inter-ploidy derived progenies (Chapter 2).

Using advanced molecular and cytological techniques, this study has re-investigated the usefulness of hexaploid/tetraploid crosses in breeding. Knowledge developed through this project may help to overcome several complications that plant breeders face to

employ hexaploid/ tetraploid cross as a potential method for bread and durum wheat improvement. Below I summarise the key findings from each of the research questions investigated for this PhD followed by the future directions of my study.

Chapter 3: At which generation do hexaploid/tetraploid derived progenies stabilise the univalent D-genome chromosomes?

Our investigation has indicated that the generation at which the D-genome stabilises depends on the parents used in the initial cross. For example the 2WE25/950329 cross retained univalent D-genome chromosomes even after five generations, whereas, other crosses had eliminated D-genome chromosomes completely in the second or third generation of selfing. From this study, it can be concluded that selfing F_2 lines that retain intermediate D-genome chromosomes may not be helpful for genome stability. It is worth considering back-crossing F_2 derived populations with bread or durum wheat to retain or eliminate the D-genome chromosomes, respectively.

Chapter 4: Are reciprocal tetraploid/hexaploid crosses worth considering for durum wheat improvement?

It has been suggested that using the higher ploidy level parent as the maternal parent in a polyploid cross will improve the success rate in relation to seed set and germination. However, we wanted to determine whether there was an advantage in producing tetraploid/hexaploid crosses and thus investigated differences in A and B genome inheritance and D genome retention between a hexaploid/tetraploid and its reciprocal cross.

The result was that the reciprocal tetraploid/hexaploid and hexaploid/tetraploid cross both retained atleast one copy of all seven unique D-genome chromosomes to being completely absent in the subsequent F_2 generation. Both crosses hexaploid/tetraploid (2B,3A,3B) and tetraploid/hexaploid (4A) inherited a significant proportion of durum A and B genome alleles. As there were no huge differences observed between the crosses we concluded that it would be more feasible to use hexaploid/tetraploid crosses in durum breeding as these produce a better seed set.

Chapter 5: Can hexaploid/tetraploid hybridisation help to transfer the introgressed 2G segment from bread wheat into durum wheat?

The inheritance of the 2G segment varied significantly among the three crosses made with the 2G donor Sunguard used as the maternal parent. Sunguard/Caparoi crosses inherited the 2G segment in the expected Mendelian ratio 1: 2: 1 (maternal, heterozygous, paternal) whereas the remaining crosses Sunguard/Hyperno and Sunguard/WID802 had a strong preference to inherit the durum 2B chromosomal segment. As the 2G segment was inherited in the progeny of these crosses we could conclude that hexaploid/tetraploid crosses could be employed to transfer the introgressed 2G segment from bread wheat into durum wheat. However, a strong correlation between the introgressed 2G segment and D-genome retention was observed. As only a small number of progeny had the 2G segment and an incomplete D chromosome set, the introduction of the 2G segment into a durum background may not be easy. It is suggested that large populations of these crosses are produced to obtain at least some progeny with the 2G segment and no D chromosomes.

Chapter 6: Maternal inheritance of cytoplasmic genome in a pair of hexaploid/tetraploid and tetraploid/hexaploid cross

Understanding how nuclear and cytoplasmic genome interacts in inter-ploidy crosses is important in thedevelopment of successful interploidy crosses. Maternal inheritance of the cytoplasmic genome is critical for the growth and development of interploidy progenies for a viable subsequent generation. This study identified heteroplasmic regions of mitochondrial genes in bread and durum wheat. SNP's consistent across four bread and four durum wheat cultivars were identified. This was confirmed by both forward and reverse sequencing of these regions across the cultivars. This study concluded that there are nucleotide differences in the cytochrome maturation gene which can be used to identify bread or durum wheat mitochodnrial inheritance.

Future directions

There are a number of research areas that could be further investigated based on the outcomes generated from this Ph.D. study. The interploidy lines (Chapter 3 and 4) developed during this study are from crosses with a hexaploid parent that has partial crown rot resistance. Thus field trials could be conducted, to evaluate the established interploidy lines and to select lines which have crown rot resistance and could be used

in the durum breeding program. This would be valuable as current commercial durum varieties are very susceptible to this disease. Similarly other important traits present on the *T. timopheevii* (2G) segment were introgressed into current durum lines (Chapter 5). Interploidy lines from these crosses could be further assessed for disease and/or quality characteristics of importance to durum breeding. Durum lines are known to have better nematode tolerance than most of the current bread wheat varieties (Sheedy 2015). Thus some of the interploidy lines which have all seven D chromosomes may be valuable as hexaploid sources in the wheat breeding program.

An extensive lab based cytological investigation could be carried out (seed materials from Chapter 3 and 5) to study the univalent D-genome retention from one generation to another. This Ph.D. study only looked into the D-genome retention of somatic cells from the root tips of the selected lines and did not investigate the meiotic cell division. Both mitotic (root tips) and meiotic (pollen mother cells) cell division could be documented in the same lines, to understand the transmission of univalent D-genome retention from somatic cells (diploid) to reproductive cells (haploid). Perhaps this could open up new insights into how univalent or telocentric D chromosome divide in mitotic and meiotic cells that contain unbalanced chromosome numbers and how these chromosomes are able to pass through cells from one generation to another.

Protocols are available for mitochondrial DNA isolation, however, separation and purification of mitochondrial DNA from chloroplast and nuclear DNA is a highly demanding and tedious process. Development of a simple and rapid protocol to isolate and purify mitochondrial DNA from genomic DNA would be useful to get quality mitochondrial DNA sequences of current bread and durum wheat cultivars (Chapter 6). High quality whole mitochondrial genome sequences could be used to investigate the inheritance of paternal sequences and its impact on growth and pollen development (cytoplasmic male sterility) in hexaploid/tetraploid wheat lines.

Conclusion

There is a significant opportunity for both bread and durum wheat breeders to expand the research towards the area of inter-ploidy hybridisation. Hexaploid/tetraploid derived lines are already showing promising results for the level of tolerance to crown rot disease (Martin et al., 2013). Implications of this study may help in increasing the

level of crown rot resistance in durum wheat cultivars which will be a major break-through for the Australian durum industry. As hexaploid/tetraploid lines also exhibit variations in grain size, weight and length there could be room for improving lines for seed yield and quality characters. Initiating research projects on hexaploid and tetraploid wheat crosses for incorporating yield and quality characters, nematode and saline tolerance from either bread or durum wheat will support researchers to increase wheat production. Thus developing hexaploid/tetraploid wheat crosses could be one of the potential ways to address food production for feeding the future population.

References

Eberhard FS, Zang P, Lehmensiek A, Hare RA, Simpfendorfer S, Sutherland MW (2010) Chromosome Composition of an F_2 *Triticum aestivum* X *T.turgidum spp. durum* cross analysed by DArt markers and MCFISH. Crop and Pasture Science 61:619-624

Friebe B, Jiang J, Raupp W, McIntosh R, Gill B (1996) Characterization of wheat-alien translocations conferring resistance to diseases and pests: current status. Euphytica 91 (1):59-87

Kalous J, Martin J, Sherman J, Heo H-Y, Blake N, Lanning S, Eckhoff J, Chao S, Akhunov E, Talbert L (2015) Impact of the D genome and quantitative trait loci on quantitative traits in a spring durum by spring bread wheat cross. Theoretical and Applied Genetics:1-13

Kihara H (1925) Weitere Untersuchungen über die pentaploiden Triticum-Bastarde. I. Japanese Journal of Botany 2:299-305

Kihara H, Lilienfeld F (1949) A new synthesized 6x-wheat. Hereditas 35 (S1):307-319

Martin A, Simpfendorfer S, Hare RA, Eberhard FS, Sutherland MW (2011) Retention of D genome chromosomes in pentaploid wheat crosses. Heredity 107 (4):315-319. doi:10.1038/hdy.2011.17

Martin A, Simpfendorfer S, Hare R, Sutherland M (2013) Introgression of hexaploid sources of crown rot resistance into durum wheat. Euphytica 192:463-470.

Sheedy, J., Mckay, A., Lewis, J., Vanstone, V., Fletcher, S., Kelly, A., et al. (2015). Cereal cultivars can be ranked consistently for resistance to root-lesion nematodes (*Pratylenchus thornei* & *P. neglectus*) using diverse procedures. Australasian Plant Pathology. 44, 175–182. doi: 10.1007/s13313-014-0333-4