DEVELOPMENT OF GENETIC AND GENOMIC RESOURCES FOR HYBRID WHEAT (*Triticum aestivum* L.) DEVELOPMENT IN THE US GREAT PLAINS

A Dissertation

by

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ABSTRACT

Hybrid wheat offers promises to break the yield stagnation in global wheat productivity. Studies were conducted in Texas, Nebraska and the International Maize and Wheat Improvement Center (CIMMYT) in Mexico to develop genetic and genomic resources needed for development of hybrid wheat. Hybrids developed from elite winter wheat lines of the University of Nebraska Lincoln and Texas A&M University wheat breeding programs were evaluated across Texas in 2016 and 2017. The grain yield data was used to estimate heterosis and combining ability of parents. The heterosis estimates were promising with commercial heterosis ranging from -78.3 to 20.4% in 2016 and -32.9% to 6.2% in 2017. General combining ability (GCA) variance was significantly higher than zero whereas specific combining ability (SCA) variance was not. A set of hybrids with high to low yield potential were advanced to F_2 stage evaluation. They were planted in six locations across Texas and Nebraska in 2017 and 2018 to test the possibility of using F_2 yield and heterosis in supplementing selection decisions of F_1 hybrids. The hybrids exhibited positive heterosis at F_2 stage as well. A comparison of heterosis estimates between F_2 and F_1 stage revealed that F_2 heterosis was highly indicative of superior F_1 performance. A population of 299 recombinant inbred lines (RILs) were developed at CIMMYT to map fertility restoration and develop molecular markers for marker assisted selection (MAS) of *Rf* genes. The RILs were characterized for their fertility restoration capacity in a series of field trials across three locations in Mexico. The results indicated the presence of known fertility restorer genes *Rf3* and *Rf4* and a minor effect quantitative trait locus (QTL) in the restorer line. Kompetitive allele specific PCR (KASP) assays were developed using markers tightly linked to major gene *Rf3* and validated in an independent population. The parents with GCA estimates and hybrid yield can be used to develop heterotic pools whereas F2 testing provide a cost effective way of

evaluating hybrids in replicated trials. The KASP assays developed can be used for MAS of *Rf* genes. The genetic and genomic resources developed in these studies can serve as valuable assets in developing wheat hybrids for US Great Plains.

DEDICATION

This dissertation is dedicated to my parents, Dr. Bishnu Bilas Adhikari and Bimala Adhikari for making all the sacrifices to provide me quality education, and to my loving wife Pratibha Acharya for all the encouragement and constant support throughout the highs and lows of this PhD journey.

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NOMENCLATURE

CHA	Chemical Hybridizing Agent
GCA	General Combining Ability
SCA	Specific Combining Ability
MET	Multi-Environment Trials
RF	Restorer of Fertility
QTL	Quantitative Trait Loci
CIM	Composite Interval Mapping
MAS	Marker Assisted Selection

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CHAPTER I

INTRODUCTION AND REVIEW OF LITERATURE

1.1 Introduction

Wheat (*Triticum aestivum* L.) is the most widely grown food crop of the world with 771.7 million tons grown in 2018 million hectares of land area worldwide (FAOSTAT, 2017). Wheat provides 20% of the daily protein and food calories for 4.5 billion people (FAOSTAT, 2017). Wheat is increasingly becoming a more important food crop due to changes in food preferences of people in different parts of the world where urbanization and industrialization is rapid. A wheat based diet is more adopted now at the expense of other cereals such as rice (*Oryza sativa* L.), millet (*Pennisetum glaucum* L.) and sorghum (*Sorghum bicolor* (L.) Moench) in Asia and Africa (Shewry and Hey, 2015). In the western diet, wheat serves as a major provider of dietary fiber (Shewry and Hey, 2015).

In the United States, wheat is the third most important commodity after maize and soybeans in terms of acreage, production and gross farm receipts (USDA ERS, 2019). Globally the United States produces the third highest quantity of wheat (58.7 million tons) after China and India (FAOSTAT, 2017). However, the acreages of wheat are rapidly declining, and it was the record lowest in the 2017-18 season at 47 million acres (USDA ERS, 2019). This has been attributed to lower relative returns for wheat in comparison to other crops and increased competition in the global market from Russia and European Union. Wheat is still one of the major agricultural commodity exports of United States. In 2019, 27.2 million metric tons of wheat worth over 5.5 billion US dollars were exported.

Wheat yield gains, including those representing the US Great Plains, have not been rapid enough to meet the need of 9.7 billion people by 2050 (Graybosch and Peterson, 2010; Ray et

al., 2013). The wheat yield gain per year needed to meet the projected demand by 2050 is 1.9% per year whereas it has been stagnant at 0.9% since the 1990s (Graybosch and Peterson, 2010; Ray et al., 2012). Meeting this required yield gains per year is even more challenging in the face of climate change and more so in areas where wheat yields are already low (Asseng et al., 2013). Hybrid wheat breeding represents an opportunity to address this problem and enhance the yield stability in marginal environments where the slow gain in yields has been especially acute (Ray et al., 2012; Reynolds et al., 1996). Presently, hybrid wheat is planted in some parts of Europe, China and India, and it occupies less than 1% of the global wheat production area (Gowda et al., 2012; Kempe et al., 2014). In the US, hybrid wheat breeding and research has been conducted since the 1950s, and compared with commercial pure-line cultivars, higher yields and stability across environments have been reported (Bruns and Peterson, 1997; Koemel et al., 2004).

1.2 Hybrid wheat: A historical overview

After yield heterosis was reported in maize and commercially exploited in the form of hybrid maize in the 1930s, scientists started exploring possibilities of creating hybrids of other economically important crops (Shull, 1948; Virmani and Edwards, 1983). The most important food crop in the early 20th century was wheat, hence hybridization efforts in wheat started early. However, developing wheat hybrids is not easy since wheat is self-pollinated with perfect flowers and has a very low natural outcrossing rate (< 5%) (Lawrie et al., 2006). The most important challenge for hybrid wheat was and still is, controlled cross pollination (Virmani and Edwards, 1983). In maize, cytoplasmic male sterility was reported as early as 1933, however, it was not used for pollination control in hybridization until much later (Rhoades and Rhoades, 1933). This discovery was very pivotal in the interest of developing wheat hybrids using cytoplasmic male sterility (CMS). Using CMS for hybrid wheat seed production started with the

reporting of CMS in *Triticum aestivum* by Kihara (1951) using cytoplasm of *Aegilops caudata* L and *Triticum ovata* by Fukasawa (1955). Later, a more workable CMS in wheat was transferred from *Triticum timopheevi* by Wilson and Ross (1962) and corresponding restorers of fertility genes (*Rf* genes) were reported by Schmidt (1962) and Livers (1964). This prompted public wheat breeding programs and private breeding companies to initiate investments in hybrid wheat research even though the CMS system was not fully functional due to imperfect restoration of fertility.

From 1960 to 1980, some hybrid wheat varieties were also released. For example, commercial hybrid wheat varieties were released by Cargill in US and Dekalb in Australia in 1981 (Singh et al., 2010). By the early 2000s, due to low wheat prices, private companies had started to lose interest in hybrid wheat research. This resulted in Cargill stopping its hybrid wheat research in 1990s, Monsanto in 2000 and DuPont/Hybrinova in 2002 (Singh et al., 2010). DuPont's hybrid wheat business was acquired by the French company Saaten Union (Asur Plant Breeding since 2019). Hybrid wheat research continued in Europe, China and India. Presently, hybrid wheat is planted in some parts of Europe, China and India, and it occupies less than 1% of the global wheat production area (Gowda et al., 2012; Kempe et al., 2014). The International Maize and Wheat Improvement Center (CIMMYT) has also had research programs in hybrid wheat that mirror the progression of hybrid wheat interests in North America. In the early 1960s, CIMMYT had started hybrid wheat research using *T. timopheevii* CMS system which was discontinued in the 1970s (Singh et al., 2010). The interest rekindled in the 1990s via use of CHAs in collaboration with Monsanto and got discontinued in the early 2000s (Rajaram, 2001; Reynolds et al., 1996). Since 2010, CIMMYT has decided to include hybrid wheat as one of its

strategic research priorities, and hybrid wheat lines produced via CMS and CHA methods are being tested in Mexico and India (Basnet et al., 2019; Singh et al., 2010).

1.3 Heterosis and grain yield

Wheat in the US Great Plains is not a particularly profitable crop as compared to maize and soybeans due to the consistently declining wheat prices since the 1970s (Vitale et al., 2019). Higher yields due to hybrid wheat might attract farmers in the US Great Plains to plant more wheat; however, the yield heterosis must be enough to offset hybrid seed costs and make additional profit to provide impetus for farmers to adopt hybrid wheat. (Angus) 1997 estimated that a commercial heterosis of about 5% is needed for hybrid wheat to become economically viable in comparison with the best line bred variety. These numbers might now be a little higher considering these estimates were made decades ago, and wheat global prices have not increased as much. Similarly, Pickett and Galwey (1997) concluded that 6-34% of mid-parent heterosis is needed for the commercial success of hybrid wheat. A general consensus among the seed companies and hybrid wheat breeding programs is that for the commercial success of hybrid wheat in North America, a yield advantage of 10-15% is required. In Western Europe where hybrid wheat has gained over 500,000 hectares over the past decade, hybrid wheat offers a yield advantage of about 10% over the best pureline commercial wheat varieties (Longin et al., 2013).

Grain yield heterosis in wheat has been the interest of researchers as early as 1935 (Pal and Alam, 1938). The interest continued in response to progress made in hybrid wheat research, commodity prices and research investment into the 1960-1970 period (Briggle, 1963; Hermsen, 1960; Johnson and Schmidt, 1968; Knott, 1965) and the 1990s to 2000s (Barbosa-Neto et al., 1996; Borghi and Perenzin, 1994; Dreisigacker et al., 2005; Shamsuddin, 1985; Uddin et al., 1992). The earlier experiments likely had inflated, or imprecise estimates of heterosis since they

were conducted using small number of hybrids, and sometimes in hill plots because of the difficulty in producing seed (Dreisigacker et al., 2005). Recent studies were conducted using a higher number of hybrids and in yield plots and provide more precise estimates of heterosis in hybrid wheat (Barbosa-Neto et al., 1996; Dreisigacker et al., 2005). More recent ones in which hybrid seed was produced using CHA's have larger sample sizes, precise estimates of grain yield heterosis and also represent the current practices of growing wheat (Basnet et al., 2019; Gowda et al., 2010; Gowda et al., 2012; Zhao et al., 2015).

In the US Great Plains, Bruns and Peterson (1997) reported an average of 0.454 t ha^{-1} or 10.8% yield advantage of hybrids over purelines in preliminary yield trials. In addition, they reported an average of 0.652 t ha^{-1}, or 13.5% higher yield by hybrids in the advanced yield trials in Agripro Standard Variety Trials and USDA-ARS Southern Regional Performance Nurseries from 1990 to 1995. Similarly, Koemel et al. (2004) reported a yield advantage of 10.9 % in Oklahoma Variety-Hybrid Performance Nursery from 1975 to 1995. Cisar et al. (2002) reported a yield advantage of 0.26 – 0.45 tons ha^{-1} as compared to commercial checks based on 335 hard red spring wheat hybrids evaluated at one location in North Dakota in 1996 and 254 hybrids evaluated in two locations in Pacific Northwest in 1997. Hybrid wheat offers better yield advantages compared to purelines in low input environment as well. In a survey done in India over a period of five years from 2001 -2005 by a Maharastra based company Mahyco, hybrid wheat provided a yield advantage of over 0.9 ton ha^{-1} on an area of 16000 – 23000 ha of small holder farmers field (Matuschke et al., 2007).

Most of the early studies of heterosis in hybrid wheat focused on only mid-parent heterosis (Pal and Alam, 1938; Shamsuddin, 1985). Knott (1965) reported presence of high-parent heterosis but no commercial heterosis in the seven hybrid genotypes that he evaluated in

comparison to ‘Thatcher’. Uddin et al. (1992) studied all three types of heterosis, mid-parent, high-parent and commercial heterosis, in Australian spring wheat. However, the experiment was conducted in hill plots and the heterosis estimates are most likely inflated. The study by Barbosa-Neto et al. (1996) was one of the most reliable early hybrid wheat experiments since they evaluated 722 soft red winter wheat hybrids in multiple years and locations in experimental yield plots rather than hill plots. They reported mid-parent heterosis in the range of -20 to 57% and high-parent heterosis in the range of -22 to 47%, but commercial heterosis was not studied or reported. The parents for these hybrids were randomly selected, hence the heterosis estimates might be lower than in studies where some consideration is given to select genetically dissimilar parents to exploit heterosis. Dreisigacker et al. (2005) examined heterosis in a set of 112 hybrids developed from widely successful CIMMYT wheat varieties, and some Chinese lines over two years and reported -15.33 to 14.33 % mid-parent heterosis; whereas, no positive high parent heterosis and commercial heterosis was observed. Liu et al. (1999) also reported low mid-parent heterosis in Chinese spring wheat from an experiment evaluating 41 hybrids. Dreisigacker et al. (2005), based on their results, concluded that research investment is not justified in hybrid wheat because of the low amount of heterosis. However, the hybrid wheat research resumed in CIMMYT almost a decade after that in 2012 with promising results (Basnet et al., 2019). In a most recent publication from CIMMYT hybrid wheat breeding program involving 1888 experimental hybrids and 685 parents, grain yield heterosis was on average 0.43 to 0.68 t ha^{-1} or 7.5 to 9.5%, which is very promising.

More recent studies have reported higher estimates of heterosis (Gowda et al., 2010; Gowda et al., 2012; Zhao et al., 2015). Zhao et al. (2015) produced 1604 single cross hybrids using 135 diverse winter wheat lines adapted to Central Europe and evaluated them in 11 field

trials. In their study, a total of 97 hybrids outperformed the best commercial check ‘Tobak‘ with a yield advantage as high as 1 t ha^{-1} (Zhao et al., 2015). Similarly, Gowda et al. (2012) evaluated 940 winter wheat hybrids developed by the French hybrid cereal breeding company Saaten-Union in four experiments in France and reported positive commercial heterosis of about 4-5% in two out of four experiments. The experimental hybrids evaluated in that trial came from crossing 334 female lines and 114 male lines in incomplete factorial crosses. More than 150 lines evaluated across four trials performed better than the best commercial check in respective experiment. The best hybrid yielded 1 t ha^{-1} higher than the best commercial check in two of the experiments. In durum wheat (*Triticum turgidum* ssp. *durum*) hybrids also have been shown to out yield best checks by more than 1 t ha^{-1} or 22 % (Gowda et al., 2010).

1.4 Heterotic pool development

Heterotic pools are divergent groups of germplasm which are paramount for commercial success of hybrid crops since crossing germplasm from these groups with each other maximizes heterosis (Falconer and Mackay, 1996; Melchinger and Gumber, 1998). The importance of heterotic pools in the success of hybrid breeding is quite exemplified in hybrid maize and hybrid sorghum breeding (Melchinger and Gumber, 1998; Menz et al., 2004; Reif et al., 2005). In presence of heterotic pools, reciprocal recurrent selection schemes can be used to improve genetic mean of either pools while minimizing genetic variance within pool and maximizing genetic distance between pools such that crosses between these pools exhibit heterosis (Falconer and Mackay, 1996). This allows the breeders to maximize resources in hybrid breeding, most importantly in making parental selection for hybrid cross evaluations.

In the past, when molecular markers were not available, attempts at assigning promising parental lines into heterotic groups were based on estimates of combining abilities calculated via

phenotypic performance data (Kronstad and Foote, 1964; Shamsuddin, 1985). This strategy is very useful when appropriate mating designs are used to get accurate estimates of combining abilities. Identifying heterotic pattern based on estimated combining abilities is still practiced (Adhikari et al. 2020.; Easterly et al. 2020.; Gowda et al., 2010; Gowda et al., 2012). However, this strategy is limited in scope because only a small number of lines can be used in these mating designs and phenotypic evaluations for combining ability estimation are very expensive. Another strategy is to use molecular markers to determine the genetic distance of parental lines and assign them to heterotic groups based on that information (Barbosa-Neto et al., 1996; Dreisigacker et al., 2005; Liu et al., 1999). Zhao et al. (2015) proposed a strategy for identifying heterotic pattern in wheat via use of dense molecular markers in a genomic prediction scenario. Zhao et al. (2015) evaluated 1604 hybrids of European winter wheat derived from 135 parents in field trials and used the genotypic and phenotypic data from this group to predict 9045 hybrids not tested in the field using genomic prediction. Finally, the genomic information and the predicted hybrid performance was used to divide parental lines into heterotic groups. Based on simulation studies, they also concluded that for hybrid wheat breeding program in Central Europe, heterotic groups consisting of only 16 individuals can guarantee long-term success in improving grain yield performance. A long-term strategy in this scenario would be to continue evaluation of new lines for combining abilities and start assigning them to these predefined heterotic groups based on combining ability estimates. Recently, Technow (2019) showed some empirical evidence for assigning parents to heterotic pools via use of F_2 performance data as a training dataset in a genomic prediction model. He argues that in self-pollinated crops where heterotic pools are not predefined, use of F_2 data is very sensible due to scientific as well as practical considerations. A similar study is underway in US Central Plains using germplasm from Texas A&M and

University of Nebraska Lincoln wheat breeding programs. The research done for this dissertation is a part of that study.

1.5 Hybrid seed production

1.5.1 Seed production using chemical hybridizing agents

The chemical hybridizing agents (CHA) are chemical gametocides that when sprayed on female parents in the crossing block cause pollen sterility (Figure 1). Using a CHA any normal wheat line can be converted into a female line. The female line when crossed with a male line produces hybrid wheat whereas seed multiplication of either male or female lines can be done by allowing them to self-fertilize.

The possibility of using chemicals as hybridizing agents was reported as early as 1953 (Hoagland et al., 1953). Later, Indian scientists explored the possibility of using maleic hydrazide at different concentration to induce pollen sterility in a number of economically important crops including wheat (Chopra et al., 1960). Maleic hydrazide, even in low concentrations would severely damage the wheat plants in addition to causing sterility.

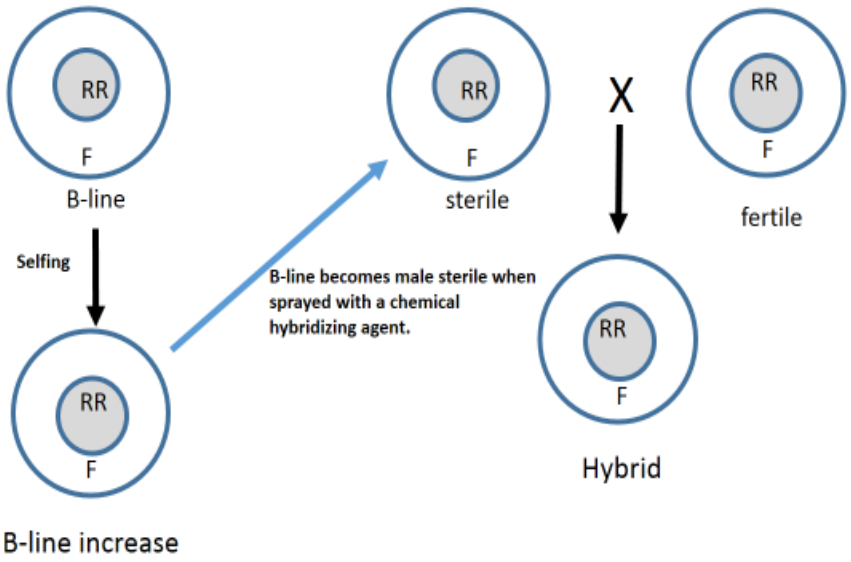

Figure 1 Hybrid wheat production using a chemical hybridizing agent which causes a normally male fertile line to become male sterile. Adapted from (Easterly 2017)

Other alternatives to maleic hydrazide such as 2 Chloroethylphosphonic acid (trade names: Etherel and Ethepon) were reported by scientists in the US in the 1970s (Rowell and Miller, 1974; Rowell and Miller, 1971). However, these alternatives also had phytotoxic effects, resulting in poor female receptivity and their performance was very unstable across different environmental conditions (Cisar et al., 2002). These compounds were developed for other purposes such as anti-lodging or height reduction agents and were not quite suitable for hybrid seed production. Later, in the 1980s and 1990s, CHAs specific for hybrid seed production were developed and tested. In 1984, a CHA of undisclosed chemical composition called WL 84811was successfully used to produce hybrid seed with higher than 80% hybrid purity (Borghi et al., 1988; Morgan et al., 1989). Another CHA called SD 84811 was tested by Pioneer in 1985, which caused severe phytotoxic effects and hybrid seed produced via its use was 50% less compared to CMS system (Howey et al., 1988). In the 1990s, another CHA called the SC 2053

was tested in France which also failed commercially (Streiff et al., 1997). All of these CHAs worked best under a narrowly defined set of environmental conditions such as a very limited application window, temperature constraints and were restricted to limited genotypes (Cisar et al., 2002). Monsanto's CHA "Genesis" and DuPont/Hybrinova's "Croisor" were some of the better CHA's extensively used to develop experimental hybrids in US, Australia and Europe by companies such as Dekalb, Pioneer, Cargill and Monsanto in the 1970s to early 2000s (Cisar et al., 2002; Pickett, 1993).

Since the early 2000s, a CHA called Croissor 100® has been successfully used by Saaten Union (https://www.asur-plantbreeding.com/). Some of the commercial hybrids developed via use of this CHA gained significant acreage (~10%) in western Germany and Northern France. This CHA has also been successfully used for experimental seed production by academic research programs in Germany and US (Adhikari et al. 2020; Easterly et al. 2020.; Gowda et al., 2010; Gowda et al., 2012). In Nebraska, Easterly (2017) reported 75-80% purity of hybrid wheat seed produced via use of Croissor 100® in winter wheat.

1.5.2 Seed production using cytoplasmic male sterility

Cytoplasmic male sterility (CMS) in plants is a result of expression of mitochondrial and chloroplast proteins in the tapetum layer of anthers, which provides nutrients and structural integrity to developing pollen grains (Schnable and Wise, 1998). This sterility can be overcome by the effects of fertility restorer nuclear genes that interfere with the expression of these proteins from chroloplasts and mitochondria in the anthers. This combination of biologically induced sterility and fertility restoration via nuclear genes has been extensively exploited in crop plants to produce hybrid seeds (Schnable and Wise, 1998). To use this method in hybrid seed production, three types of lines are needed. The first one is the female line that has the sterile

cytoplasm, a.k.a. A line. The second line is the alloplasmic line of the female line called a B line, which has same nuclear genes as the A line but a normal cytoplasm. The third line is a male line, a.k.a. R line that has the corresponding restorer of fertility genes in the nucleus. When A and R lines are crossed hybrid seed is produced; whereas when A and B lines are crossed, a CMS A line is produced. The B line is needed because A line cannot self-propagate because of the CMS. This method of hybrid seed production is often called the three-line system or XYZ system (Figure 2).

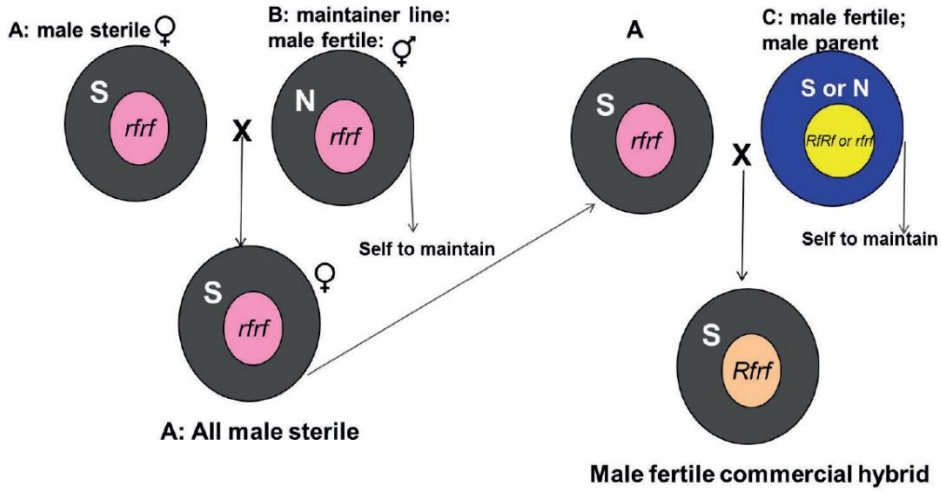

Figure 2 A three-line system of hybrid seed production that utilizes cytoplasmic male sterility and fertility restoring genes. Reprinted from (Lin et al. 2013)

Interests on using cytoplasmic male sterile wheat for hybrid seed production started with the reporting of cytoplasmic male sterility (CMS) in *Triticum aestivum* by Kihara (1951), using cytoplasm of *Aegilops caudata* L., and in *T. ovata* by Fukasawa (1955). Later, CMS wheat was developed using cytoplasm of *Triticum timopheevi* by Wilson and Ross (1962), which was found to be most suitable for hybrid seed production (Wilson and Driscoll 1982). Fertility restoring

lines for CMS wheat lines with *T. timopheevi* was first reported by Schmidt (1962) and later by Livers (1964). Livers (1964) reported that fertility restoration was possible by transferring *Rf* genes from *T. timopheevi* to common wheat and using it as a male parent. He reported that fertility restoration was conditioned by two dominant restorer genes *Rf1* and *Rf2*, but the chromosomal location of them was unknown. This initiated great interest on genetic mapping of these fertility restorer (*Rf*) genes by using monosomic lines.

Robertson and Curtis (1967) conducted the pioneering work of mapping *Rf* genes using monosomic lines. They mapped the *Rf* gene *Rf1* in chromosome 1A in a line developed by Livers (1964) and reported other modifier genes in 1B, 2A, 3D, 6A and 6B chromosome arms. A similar study was done by Yen et al. (1969) in which they mapped *Rf* genes in varieties 'Canthatch', 'Dirk' and 'Karn' as restorer parents with CMS female lines having *T. timopheevi* cytoplasm. A major gene *Rf1* on chromosome 1A and minor gene *Rf4* was reported on chromosome 7D in Dirk; a major gene *Rf2* and minor gene *Rf3* in 6B and 6D, respectively in Canthatch and other genes in 1A and 6B in Karn (Yen et al. 1969). Tahir and Tsunewaki (1969) also conducted a monosomic analysis with *Triticum spelta* var. *duhamelinum* in 'Chinese Spring' background and found a major *Rf* gene in 1B and designated it *Rf3*, the nomenclature of which contradicts with that of Yen et al. (1969). Later, Bahl and Maan (1973) mapped fertility restorer genes in six sources of fertility restorer genes using monosomic analysis. The fertility restorer sources used were R1-Lee, R2-Sonora 64, R3, R4, R5 and 'Primepi'. Several other researchers have previously used these lines to map fertility restorer genes (Bahl and Maan 1973). Two *Rf* genes each were reported on R1-Lee, R3, R4 and Primepi whereas three genes were reported on R2-Sonora 64 and R5. *Rf* genes were reported on 1A and 7D in R1-Lee, R2-Sonora 64, R3, R4 and R5. R2-Sonora 64 had an additional gene on 6B and R5 had an additional gene on 7B.

Primepi had new set of genes on 1B and 5D chromosomes. Maan et al. (1984) mapped fertility restorer genes in the R-line (R113) with *T. timopheevii* cytoplasm in a set of monosomic test cross families. They mapped major genes on chromosome 1A and 6B and other modifier genes on 7B, 1D, 4D, 5D and 7D. Considering that the R-line R113 used by Mann et al. (1984) had recessive *Rf* genes, other than *Rf1*(1A) and *Rf4* (6B) and other modifier genes, Du et al. (1991) studied R113 derived lines in monosomic and disomic populations. Du et al. (1991) could not find the recessive restorer genes as they expected and concluded that fertility restoration is conditioned by several modifier genes all across the wheat genome. Similarly, *Rf* gene mapping efforts were being carried out on CMS systems other than *T. timopheevi*. In a hexaploid wheat cultivar 'Norin 26' with *Aegilops crassa* cytoplasm, a major Rf gene was mapped in chromosome 7B by Murai and Tsunewaki (1994).

After molecular markers were accessible in the 1990s, mapping efforts were carried out using molecular markers. Genes on the line R113 used by Maan et al. (1984) was mapped by Ma and Sorrells (1995) using RFLP markers (Table 1). They mapped the genes Rf3 and Rf4 in 1BS and 6BS, respectively. Rf3 was also mapped by Kojima et al. (1997) in 'Chinese spring' at chromosome 1BS using RFLP markers. Ahmed et al. (2001) also used RFLP markers to map QTLs in Chinese Spring and found major QTL in 1BS and minor QTLs in 2A, 4B and 6A. Similarly, *Rf3* was also mapped to chromosome 1B by Zhou et al. (2005) using Simple Sequence Repeats (SSR) markers in two restorer lines R18 and R9034 whose pedigrees trace back to cultivar Primepi and a derivative of restorer line from Kansas.

Sinha et al. (2013) reported a new fertility restorer gene in chromosome 1DS in a fertility restoring line PWR4099 using *T. timopheevi* sterile cytoplasm. The mapped gene was named *Rf8*. Many studies have tried to map restorer genes in previous sources of fertility restoration

using modern mapping methods and next generation sequencing markers (Geyer et al. 2016, Geyer et al. 2017, Wurshum et al. 2017). Fertility restorer genes in cultivar Primepi was previously mapped by Bahl and Maan (1973) using monosomic analysis in chromosome 1B and 5D and the two-gene inheritance was confirmed by Miller et al. (1974) by looking at segregation ratios (Table 1). The genes in same restorer line were mapped by Geyer et al. (2016) using modern SNP markers. In contrast to previous findings that fertility restoration is conditioned by two genes in Primepi (Bahl and Maan 1973; Miller et al. 1974), Geyer et al. (2016) found that a single gene conditions full fertility in Primepi and it was *Rf3* that mapped to 1BS.

Table 1 Restorer of fertility genes mapping studies conducted for *Triticum timopheevii* cytoplasmic male sterility.

Reference	**Genes**	**CH[a]**	**Source[b]**	**Comments**
Robertson and Curtis (1967)	*Rf1*	1A	Marquis	Modifier genes reported in 1B,2A, 3D, 6A and 6B
Yen et al. (1969)	*Rf1*	1A	Dirk	
	Rf4	7D	Dirk	Reported as minor gene
	Rf2	6B	Canthatch	
	Rf3	6D	Canthatch	Reported as minor gene
Tahir and Tsunewaki (1969)	*Rf3*	1B	Spelta wheat with Chinese Spring background	The gene nomenclature contradicts with that of Yen et al. (1969)
Bahl and Maan (1973)	-	1A	R1-Lee, R2-Sonora 64, R3, R4, R5	R3, R4 and R5 were experimental lines with no names given
	-	7D	R1-Lee, R2-Sonora 64, R3, R4, R5	
		6B	R2-Sonora	
		7B	R5	
		1B	Primepi	Reported as new genes
		5D	Primepi	Reported as new genes
Maan et al. (1984)	*Rf1*	1A	R113	R113 is North Dakota line with *T. timopheevi* source of Rf
	Rf4	6B	R113	
Ma and Sorrells (1999)	*Rf3*	1B	R113	R113 is North Dakota line with *T. timopheevi* source of Rf
	Rf4	6B	R113	
Kojima et al. (1997)	*Rf3*	1B	Chinese Spring	RFLP markers
Ahmed et al. (2001)	*Rf3*	1B	Chinese Spring	RFLP markers
Zhou et al. (2005)	*Rf3*	1B	R18 and R9034	They have Primepi in their pedigree
Geyer et al. (2017)	*Rf3*	1B	Primepi	SNP markers
Wurshum et al. (2017)	*Rf3*	1B	HeTi505, R6001	Triticale lines with *T. timopheevi* cytoplasm and SNP markers

[a]Chromosome
[b]Source of restorer gene

The overall objective of the studies done as a part of this dissertation were to develop genetic and genomic resources in winter wheat for development of hybrid wheat suitable for US Great Plains. In the first study, experimental hybrids produced from 25 parents using CHAs were tested across three locations in Texas and phenotypic data was used to estimate heterosis and combining abilities of the parents. The parents include elite lines from wheat breeding programs of Texas A&M University and University of Nebraska-Lincoln. Hence, the experimental hybrids and combining ability estimates are genetic resources that might serve in developing heterotic pools in the future. In the second study, a subset of F_2 hybrids were evaluated across Texas and Nebraska with the goal of developing a foundation for using F_2 performance data as a proxy for F_1. This study is of great practical importance in current hybrid wheat breeding programs as this strategy can enable breeding programs to increase the phenotypic testing of hybrids without concerns for expenses due to hybrid seed. Finally, the objectives of the third study is to develop a functional CMS based hybrid seed production system. The specific objectives of the study are to genetically map fertility restorer genes in a promising restorer gene and develop marker platforms for MAS of restorer genes in breeding programs.

CHAPTER II

ESTIMATION OF HETEROSIS AND COMBINING ABILITIES OF US WINTER WHEAT GERMPLASM FOR HYBRID DEVELOPMENT IN TEXAS[*]

2.1 Introduction

Since the late 1990s. wheat (*Triticum aestivum* L.) yield gains, including those representing the US Great Plains have not been rapid enough to meet future global wheat production needs (Graybosch and Peterson, 2010; Ray et al., 2013). Hybrid wheat breeding represents an opportunity to address this problem and enhance the yield stability in marginal environments where the slow gain in yields has been especially acute (Ray et al., 2012; Reynolds et al., 1996). Presently, hybrid wheat is planted in some parts of Europe, China and India, and it occupies less than 1% of the global wheat production area (Gowda et al., 2012; Kempe et al., 2014). In the US, hybrid wheat breeding and research has been conducted since the 1950s, and compared with commercial pure-line cultivars, a yield advantage has been reported. In the US Great Plains, Bruns and Peterson (1997) reported an average of 0.454 t ha^{-1} or 10.8% yield advantage of hybrids over purelines in preliminary yield trials. In addition, they reported an average of 0.652 t ha^{-1}, or 13.5% higher yield by hybrids in the advanced yield trials in Agripro Standard Variety Trials and USDA-ARS Southern Regional Performance Nurseries from 1990 to 1995. Similarly, Koemel et al (2004) reported a yield advantage of 10.9 % in Oklahoma Variety-Hybrid Performance Nursery from 1975 to 1995. Despite these demonstrations of a

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hybrid yield advantage, hybrid wheat has yet to be commercially produced on significant acerage (Knudson and Ruttan, 1988). This can be partially attributed to higher costs associated with the production of hybrid seed (Cisar et al., 2002). Attempts by industry to commercialize hybrid wheat have been hindered primarily because of the lack of a cost-effective hybrid seed production process (Cisar et al., 2002). Considerable progress has been made to develop a successful cytoplasmic male sterility system and more cost-effective seed production processes (Geyer et al., 2018; Tucker et al., 2017; Würschum et al., 2017). In addition, the potential utilization of genomic prediction for predicting hybrid performance and opportunity to establish of heterotic patterns offers great promises for hybrid wheat technology (Zhao et al., 2015; Zhao et al., 2013).

An important challenge in hybrid breeding is selecting the best parents and parental combinations from a large number of possibilities to produce the highest performing hybrids (Bernardo, 2002; Gowda et al., 2012). Estimation of General Combining Ability (GCA) and Specific Combining Ability (SCA) and using these estimates to guide parental selection is a tested and proven approach for various hybrid crops (Bernardo, 2002; Isik et al., 2017). The concepts of GCA and SCA were first defined by Sprague and Tatum (1942). GCA is the average performance of a parent in a series of cross combinations producing hybrids whereas SCA is the deviation of a parent from its GCA in a specific cross combination (Bernardo, 2002; Griffing, 1956). Hence, GCA is an important consideration selecting inbred parents; whereas, SCA is important in identifying and selecting the best single cross hybrids (Comstock et al., 1949). Phenotypic selection of parents can be based on GCA and line-per-se performance (Gowda et al., 2010). Selection on line-per-se performance is based on correlations between mid-parent values and the hybrids performance. The SCA effects are of primary importance for comparing within

group crosses and for improving the overall phenotypic mean of heterotic groups; whereas, GCA effects are primarily of significance for comparing between group crosses, as has been shown theoretically and in experimental results produced using maize (*Zea mays* L.) (Melchinger and Gumber, 1998).

As demonstrated for maize, the ratio of σ^2 GCA to σ^2 SCA is useful as a means of measuring hybrid performance (Fischer et al., 2008; Reif et al., 2005). Additionally, in the absence of epistasis, σ^2 GCA is indicative of additive genetic effects, whereas, σ^2 SCA is indicative of dominance effects (Falconer and Mackay, 1996). If the lines under consideration are fully inbred, and the intensity of selection for both GCA and SCA are the same, the relative amount of improvement coming from each of these effects will be proportional to their variances (Falconer and Mackay, 1996). For example, if the ratio of σ^2 GCA to total variance is 0.8 in a particular population, then the improvement that can be made in that particular population by plant breeding is about 80%; whereas, the remaining improvement is due to SCA (Falconer and Mackay, 1996). A higher σ^2 GCA to σ^2 SCA ratio generally indicates the use of genetically dissimilar parents, which is mostly the case when heterotic patterns have been identified among parents (Reif et al., 2005). Because of the impact of σ^2 GCA compared with σ^2 SCA in the context of hybrid performance, early testing is often more effective, and superior hybrids can potentially be identified and selected based mainly on their predicted performance from GCA effects (Melchinger et al., 1987). In addition, recurrent selection can be practiced and genetic gain achieved in the respective male and female pools, if σ^2 GCA is greater than σ^2 SCA (Gowda et al. 2012). Hence, an estimation of σ^2GCA and σ^2 SCA is important in hybrid crop development and breeding, and it is useful to have a higher σ^2GCA to σ^2 SCA ratio.

Estimates of the heterosis expressed by wheat hybrids have been made over several decades (Pal and Alam, 1938) and continues (Basnet et al., 2019). However, most of these earlier estimates were based on rather non-robust experiments with hybrids planted in small hill-plots. As a consequence, the estimates were very inconsistent (Dreisigacker et al., 2005). Attempts at estimating of heterosis using methods that represent modern, wheat cultivation practices and modern elite winter wheat breeding germplasm adapted to the southern great plains of the US have not been undertaken. Moreover, there have not been recent hybrid wheat experiments conducted to estimate σ^2 GCA and σ^2 SCA and identify and exploit potential heterotic groups. In this study we estimated the heterosis, σ^2 GCA and σ^2 SCA of hybrids produced from elite winter wheat breeding lines from two large public winter wheat breeding programs at Texas A&M University (TAMU) and the University of Nebraska, Lincoln (UNL). These breeding programs breed varieties adapted to the Southern and Northern Great Plains of US, respectively. The objectives of this study were to (i) obtain estimates of mid-parent, high-parent, and commercial heterosis as well as estimates of σ^2 GCA and σ^2 SCA (iii) investigate potential associations between hybrid performance and GCA effects, (iv) examine the direct and indirect effects of yield components on hybrid wheat grain yield and, (v) determine the utility of using σ^2 GCA and σ^2 SCA to predict hybrid performance.

2.2 Materials and methods

2.2.1 Plant materials and field experiments

Hybrid evaluation trials were conducted in 2016 and 2017. Seed for these trials was produced in 2015 and 2016 from two field crossing blocks using the chemical hybridizing agent (CHA) Croisor® 100 produced by Saaten-Union (now Asur Plant Breeding, Estrées-Saint-Denis,

France). The field crossing blocks consisted of elite advanced breeding lines and released varieties from the US winter wheat breeding programs of TAMU and UNL. These lines and varieties were selected from two different breeding programs for their regional adaptation and for floral traits favoring cross pollination. Crossing blocks were planted in Lincoln, Nebraska and Greenville, Texas and consisted of 13 lines from UNL and 12 lines from TAMU, and they were purposely chosen because they potentially represent a diversity of genetic backgrounds as compared to lines from within a single breeding program. In 2015 and 2016, the crossing block consisted of a diallel design with reciprocal crosses included. A single crossing block consisted of nine female lines surrounded by a unique male line, and crossing blocks were at least 15 m distance from the adjacent crossing blocks. The female plots (1.5 x 3.3 m) in the crossing block were sprayed with Crossior 100©. The CHA performs as a male gametocide and it was sprayed before flowering at stage 34 of the Zadoks scale (Zadoks et al., 1974), which is when the immature inflorescence are about 1.5 – 1.8 cm in length. The CHA was sprayed as per manufacturer's instructions. To confirm the efficacy of the CHA a few plants in random female plots were covered with white cloth bags and seed set on the covered plants was checked at seed harvest.

The hybrids planted in 2016 and 2017 were derived from the 2015 and 2016 crossing blocks, respectively. The hybrid evaluation trial in 2016 consisted of 612 hybrids with 26 parents and 5 commercial checks and it was planted in McGregor, TX (Table 2). The experiment consisted of 780 experimental plots (1.5 x 3.3 m) with seven linear rows, laid out in an augmented randomized block design with 26 incomplete blocks. The seeding rate was set at 260 seed per m^2. Five commercial cultivars namely, 'Freeman'(PI 667038) (Baenziger et al., 2014), 'TAM 111' (PI 631352) (Lazar et al., 2004),'TAM 304' (PI 655324) (Rudd et al., 2015),

'Wesley' (PI 605742) (Peterson et al., 2001) and 'Settler CL' (PI 659690) (Baenziger et al., 2012) were randomly assigned to each incomplete block and represented pure-bred types adapted to the US Hard Winter Wheat Region. The hybrid trial in 2017 consisted of 470 hybrids, 26 parents and four commercial checks. The trials in 2017 were planted in Greenville and Bushland, TX in an augmented row-column design consisting of 600 plots with 20 incomplete blocks and 1.7 x 5 m plots. Four commercial checks, 'Ruth' (https://cropwatch.unl.edu/ne10589-ruth), Wesley, Freeman and TAM111 were assigned to each incomplete block with each check appearing only once in each incomplete block.

Freeman is a hard red winter wheat (HRWW) variety released by the Nebraska Agricultural Experiment Station (NAES) and the USDA-ARS in 2013 for its broad adaptation (Baenziger et al., 2014). It has been one of the highest yielding cultivars in statewide variety trials in Nebraska since 2013 and yielded about 3.5 - 4.9 t ha^{-1} s in four different testing regions of Nebraska in 2018 (Regassa et al., 2018). TAM 111 is a HRWW variety released by Texas A&M AgriLife Research in 2004 (Lazar et al., 2004). It is still widely grown in the Texas High Plains region and in 2018 its average yield was t 5.1 t ha^{-1} in irrigated trials (Neely, 2018). TAM 304 is a winter wheat variety released by Texas A&M AgriLife Research in 2008 and its adaptation is primarily for central Texas and Blacklands regions of Texas (Rudd et al., 2015). TAM304 was a high yielding cultivar in state variety trials in 2018 and yielded about 2.2 t ha^{-1} in the Rolling Plains, and 3.6 t ha^{-1} in the Blacklands region (Neely 2018). 'Settler CL' is a HRWW developed by NAES and USDA-ARS in 2008 (Baenziger et al. 2008). Its grain yield in 2018 was 3.1- 4.8 t ha^{-1} in state variety trials. Similarly, Wesley is a HRWW variety released by NAES in 1998 (Peterson et al., 2001) and is a consistent high yielding cultivar in NE, exhibiting average yields of 3.2 to 4.5 t ha^{-1} in state variety trials in 2018.

There was duplication of some hybrids and pure-line cultivars in experiment 1 and experiment 2. Experiment 2 included 333 hybrid entries out of 612 hybrids from experiment 1 and it included all of the parents from experiment 1.

2.2.2 Statistical analysis

Spatial Analysis of Experiment 1

The first experiment was analyzed as a randomized augmented design using incomplete blocks to control the spatial heterogeneity of the field. Two methods were tested to control spatial heterogeneity in experiment 1. The first method was a moving means analysis implemented in "mvngGrAd" package in R version 3.4 (Technow 2011) that calculates a moving average using residuals from neighboring plots. The second method involved using only the experimental design features i.e, incomplete blocks for control of spatial heterogeneity. The analysis for the second method was conducted using the following linear mixed model

$$\mu_{ij} = \mu + B_i + C_j + G_k + \varepsilon_{ijk}$$

in which μ_{ij} was the unadjusted phenotypic data of the hybrids, μ was the overall mean, B_i was the effect of $_i$th incomplete block, C_j was the fixed effect of the check, G_k was the random effect of hybrid genotypes, and ε_{ijk} was the residual.

Spatial Analysis of Experiment 2

The second experiment was analyzed using a mixed model that either accounted for experimental design features, such as incomplete blocks, rows and columns, or spatial correction models with all terms except checks fit as random effects. The analysis for single location was conducted using the following linear mixed model supplemented by spatial correction models

$$\mu_{ij} = \mu + B_i + C_j + G_k + R_l + Cl_m + \varepsilon_{ijklm}$$

in which μ_{ij} was the unadjusted phenotypic data of the hybrids, μ was the overall mean, B_i was the effect of $_i$th incomplete block, C_j was the fixed effect of the check, G_k was the random effect of hybrid genotypes, R_l was the row effect of the trial design, Cl_m was the column effect of the trial, and ε_{ijklm} was the residual. However, while testing the models, experimental design factors such as row, column and incomplete block were added in the first stage, the spatial model was added in the second stage, and if spatial models performed better than models with experimental designs only, experimental design factors were added in the third step to determine if inclusion of both spatial models and experimental design factors improved the model fit. The fit of the models was assessed by calculating AIC values and evaluating the normality of residuals.

2.2.3 Test of effects of chemical hybridizing agent on grain yield

A test to determine the carry over effects of the CHA on yield was conducted by comparing parents and checks and their CHA aided self-pollinate counterparts in the complete diallel mating design. Two groups of lines were produced for comparison. One group consisted of checks and parents, which were not treated with CHA (called selfs hereafter). The other group consisted of same checks and parental lines, which were sterilized with use of CHA and crossed with male pollinator of the same genotype. Hence, each individual crossing block included one hybrid, which was a cross between the male pollinator of the crossing block and a chemically sterilized female of the same genotype. This group of lines are the selfed lines with CHA treatment i.e., the second group (called CHA selfs hereafter). Analysis of variance (ANOVA) was conducted between these two groups (selfs and CHA selfs) to determine, if there was a significant carryover effect of CHA on seed quality in the next generation and subsequently yield. In the ANOVA, genotypes and groups as factors where lines were treatments within factor

genotypes, and year was treated as replication, since identical crossing blocks were planted in 2015 and 2016.

2.2.4 Estimation of heterosis

The experiment in 2016, consisted of a single field trial. Thus, heterosis estimation used data from the single trial. The experiment in 2017 consisted of two field trials, hence a combined analysis of both locations using homogeneous and heterogeneous error variances was also conducted to identify the best possible way to account for spatial heterogeneity. A comparison of models was done using AIC values to select the best model for obtaining yield estimates. For estimating heterosis, location specific heterosis estimates were less important for our experiment based upon the experimental objectives; hence, a combined analysis was performed using heterogeneous error variances in experiment two.

2.2.5 Tests for reciprocal differences

A test for reciprocal effects was conducted using data from both experiments and calculating an ANOVA using cross direction (cross and reciprocal cross) with crosses as factors in SAS 9.4 using PROC GLM (SAS Institute, 2013). The crosses which did not have a corresponding reciprocal cross were removed from the analysis. In addition, individual t-tests was performed between corresponding crosses and reciprocal crosses in SAS 9.4.

2.2.6 Estimation of GCA, SCA and genetic variances

Since, there was a significant overlap between genotypes between experiment 1 and experiment 2, the spatially corrected line BLUPs of yields were used to estimate a combined GCA and SCA using this model and using locations as replications.

$$Y_{ijk} = \mu + location_{(k)} + GCA_{female\ (i)} + GCA_{male\ (j)} + SCA_{female:male\ (ij)} + RGCA_{female(i)} + RGCA_{male(j)} + RSCA_{male:female\ (ji)} + error_{ijk}$$

in which Y was the phenotypic performance for the hybrid between parental lines i and j at kth environment, μ was an overall mean, $GCA_{female\ (i)}$ was the GCA effect of the ith female line, $GCA_{male\ (j)}$ was the GCA effect of the jth male line, $SCA_{female:male\ (ij)}$ was the SCA effect of crosses between lines i and j, $RGCA_{female(i)}$ was the reciprocal GCA of ith line being used as female, $RGCA_{female(j)}$ is the reciprocal GCA of jth line being used as male, $RSCA_{male:female\ (ji)}$ was the reciprocal SCA between lines i and j, and e_{ijk} was the residual. All variance components were determined by the restricted maximum likelihood method using the software ASReml-R version 3.0 (Gilmour et al., 2009).

In addition to the diallel analysis in the full dataset, three subsets of data were analyzed to look at difference in combining abilities of lines when mated with lines from within and between breeding programs. The first subset consisted of hybrids and reciprocals from TX x TX crosses (n=130, called TX dataset hereafter), the second subset consisted of hybrids and reciprocals from NE x NE crosses (n =189, called NE dataset hereafter) and finally the third subset consisted of hybrids and reciprocals from TX x TX and NE x NE crosses (n= 321, mixed dataset). Diallel analysis was conducted in these three subsets of the full dataset, using the same linear model as in full dataset to estimate combining abilities and genetic variances.

2.2.7 Path co-efficient analysis of yield components

Yield component samples were collected from the trial in Greenville 2017 from 0.6 m central linear row from the center of the plot. The samples were air dried in the greenhouse for several weeks and data were collected on dry biomass, number of productive tillers per plot (tillers that produced heads), number of seeds per spike and thousand kernel weight. Using the

plot dimensions and grain yield data from the respective plots, yield component data from the sample was extrapolated to the plot level. The plot level data was adjusted for spatial variability using the same models used for grain yield and BLUPs were extracted for all four yield components. Pearson's correlation was calculated between the yield components and grain yield BLUPs estimates in SAS 9.4. The correlation co-efficient was further partitioned into direct and indirect effects using a SAS program PATHSAS (Cramer et al., 1999; Cooper et al., 2012).

2.3 Results

2.3.1 Efficacy of chemical hybridizing agent

For testing the efficacy of chemical hybridizing agent, cloth bags were placed on female plots covering 2-3 plants, immediately after spraying the CHAs. The cloth bags have a fine mesh that prevents pollen flow from outside. Hence, seed produced on the plants inside the bag is due to selfing. At the end of the growing season, each spike inside the bags were harvested individually, and number of seeds set were recorded. The average seed $head^{-1}$ inside the bags was 5.7 in 2015 with a standard deviation of 8.1 (n=371 observations). In 2016, the average seed $head^{-1}$ was 2.6 with a standard deviation of 6.0 (n=182 observations). In 2015, 38% of the heads evaluated did not produce any selfed seed whereas that proportion was 64% in 2016 (Easterly, 2017). Hybrid crosses with high amount of selfing (> 20%) were not included in the subsequent F_1 evaluations (Easterly et al., 2019).

2.3.2 Model selection for spatial analysis

In experiment 1, only incomplete blocks were incorporated in the experimental design and row columns were absent, so we were unable to run any specific spatial models. However, the model that included experimental design features only (incomplete blocks) performed better than the statistical model that included moving means (data not shown). Hence, the adjusted

yield BLUPs from the model that included only experiment design features and not moving means was used for subsequent analyses.

In experiment 2, a range of mixed models with experimental design features, spatial models and a combination of both were tested sequentially. The order of the models tested and AIC values for each model fit are summarized in Table A1. In Bushland, the spatial variation was in the column direction and use of a mixed model with experimental design only performed the best based on the AIC values (Table A1). In Greenville, the spatial variation was in the row direction and autoregressive model in row direction had the lowest AIC value (Table A1). In the combined analysis with heterogeneous error variances also, the autoregressive model in row direction performed the best.

2.3.4 Effect of chemical hybridizing agent on wheat yield

A one-way analysis of variance revealed that there was no significant detrimental effect on wheat grain yield by use of CHA in both experiments (Figure A1).

2.3.5 Hybrid grain yield and heterosis

Hybrid grain yield in experiment 1 ranged from 671 kg ha^{-1} to 3753 kg ha^{-1} with the majority of the hybrids falling in the 1000 kg ha^{-1} to 2500 kg ha^{-1} range (Figure 3). The highest hybrid yield observed was 3753 kg ha^{-1}, which was a cross between parents from Texas and Nebraska (TX12M4063 x NE09517-1). The best performing pureline in experiment 1 was TAM 304 (3118.13 kg ha^{-1}), which performs very well in the Texas Blacklands region. As compared to other sites in 2017, McGregor was a low performing environment in 2016 most likely due to drought as indicated by the environment means (See Table 2 on p. 31); however, the highest hybrid yield was observed in McGregor.

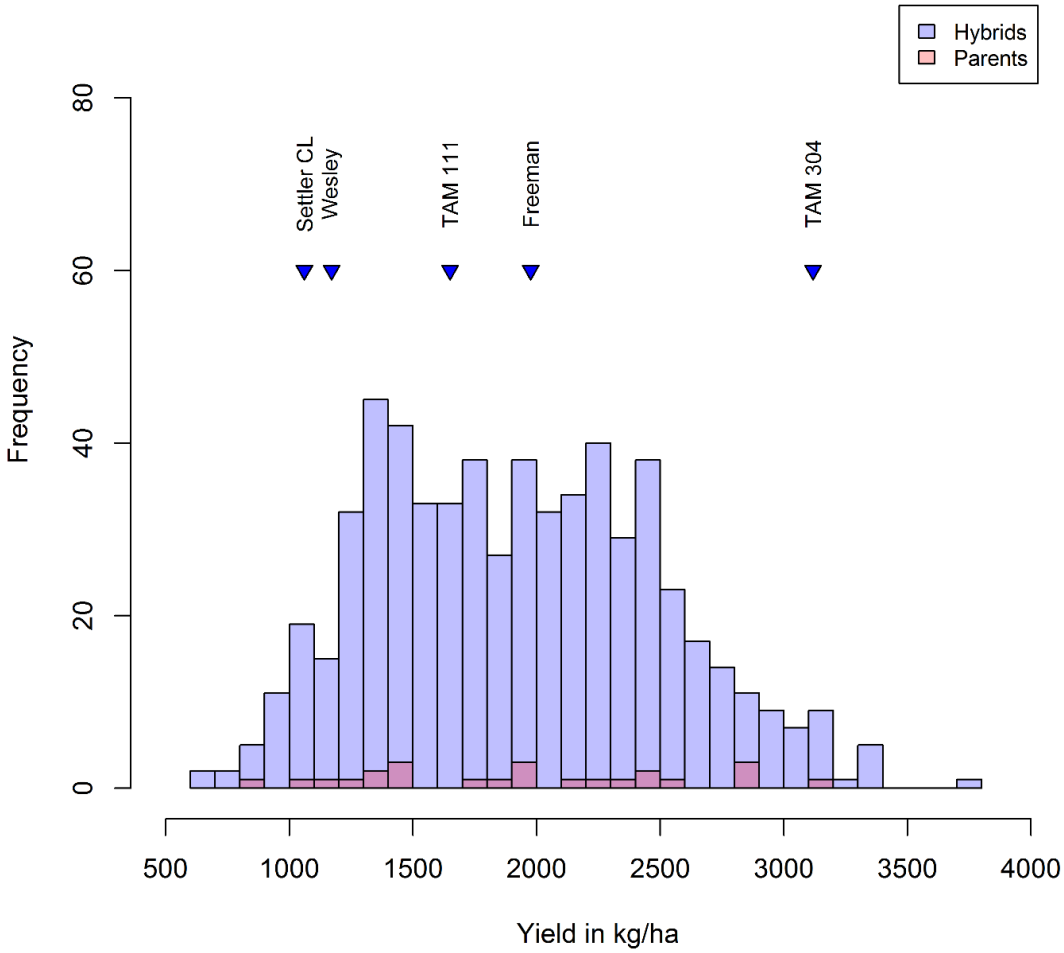

Figure 3 Yield of hybrids parents and checks in experiment 1. The yield of commercial check cultivars are represented by an inverted triangle labeled with their respective names.

Table 2 Summary of individual field trials and experiments with information about yield, heterosis estimates, performance of best check and hybrids including the common check from Texas 'TAM 111' planted across all three field trials. Experiment 1 was planted in McGregor, Texas in 2016 whereas Experiment 2 was planted in Greenville and Bushland in 2017.

	Experiment 1	**Experiment 2**		
	McGregor	**Greenville**	**Bushland**	**Combined[±]**
Hybrids	612	447	447	470
Parents	25	26	26	26
Checks	5	4	4	4
Best hybrid yield (kg/ha)	3753	2673	3707	2939
HPH range (%)	–70.41-54.31	–49.33-77.32	–27.98-31.53	–26.86-29.19
Best check	TAM 304	Freeman	Ruth	TAM 111
Best check yield (kg/ha)	3118	2478	3265	2767
Commercial heterosis (%)	–78.47-20.37	–50.06-7.86	–36.73-13.53	–32.95-6.21
[§]Yield of TAM 111 (kg/ha)	1649	2219	3223	2767
Mean trial yield (kg/ha)	1921	2012	2858	2441

[§]TAM 111 was the commercial check adapted to Texas growing conditions that was included in all three individual field trials.
[±]Combined analysis was conducted for two trials planted in Greenville and Bushland.

Fourteen hybrids yielded higher than the best check in 2016. The next best pureline after TAM 304 was a Texas advanced line (TX11D3112) which was ranked 38th in terms of grain yield and had a yield of 2881 kg ha^{-1} (Table 2). The high parent heterosis (HPH) in 2016 ranged from –78.41 to 54.31 % (Table 2; Figure 4). Commercial heterosis (CH) when compared to the best check "TAM 304" ranged from –78.47 to 20.37 %.

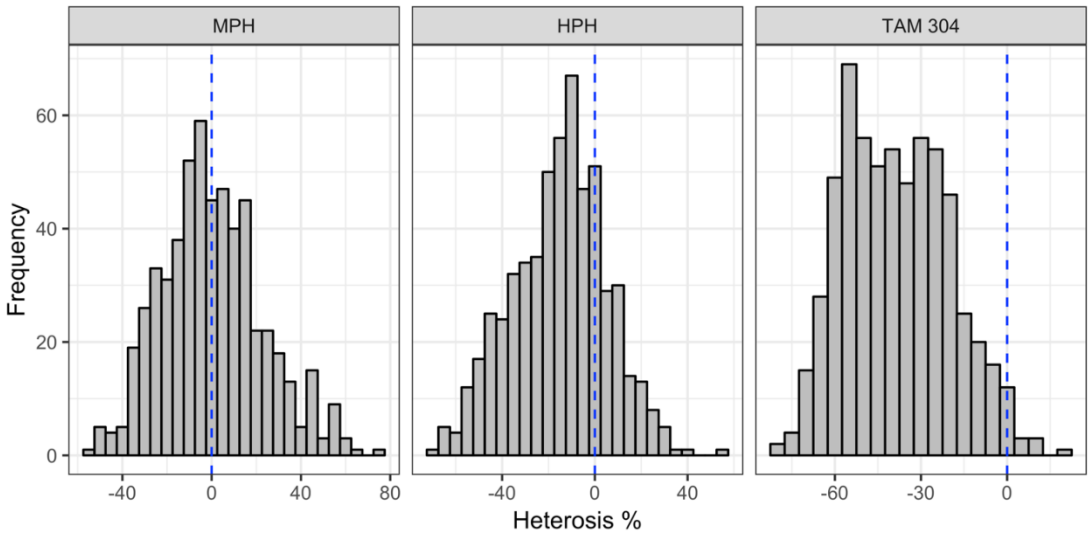

Figure 4 Mid-parent, high-parent and commercial heterosis compared to the best performing check "TAM 304" in experiment 1 represented respectively by “MPH”, “HPH” and “TAM 304”. The dotted vertical lines separate proportion of lines having positive and negative heterosis estimates.

Grain yield in experiment 2 had a much narrower range compared to experiment 1. The grain yield in experiment 2 ranged from 1855 kg ha^{-1} to 2939 kg ha^{-1} with the majority of them being in the 2300 to 2600 kg ha^{-1} range (Figure 5). The best check in experiment 2 was TAM 111, which yielded 2767 kg ha^{-1} (See Table 2 on p. 31). However, the highest yielding pureline (including parents and checks) in experiment 2 was the Nebraska NE09517-1 advanced line with yield of 2817 kg ha^{-1}. The highest yielding hybrid was NXB15-7186 resulting from a TX × TX cross with yield of 2939 kg ha^{-1}. A total of 11 hybrids and one parent yielded higher than the best check TAM 111. High parent heterosis in experiment 2 ranged from –26.86 % to 29.19 % with the majority of the estimates being negative (Figure 6). Commercial heterosis in experiment 2 with reference to best check TAM 111 ranged from –32.95 to 6.21% (Table 2; Figure 6)

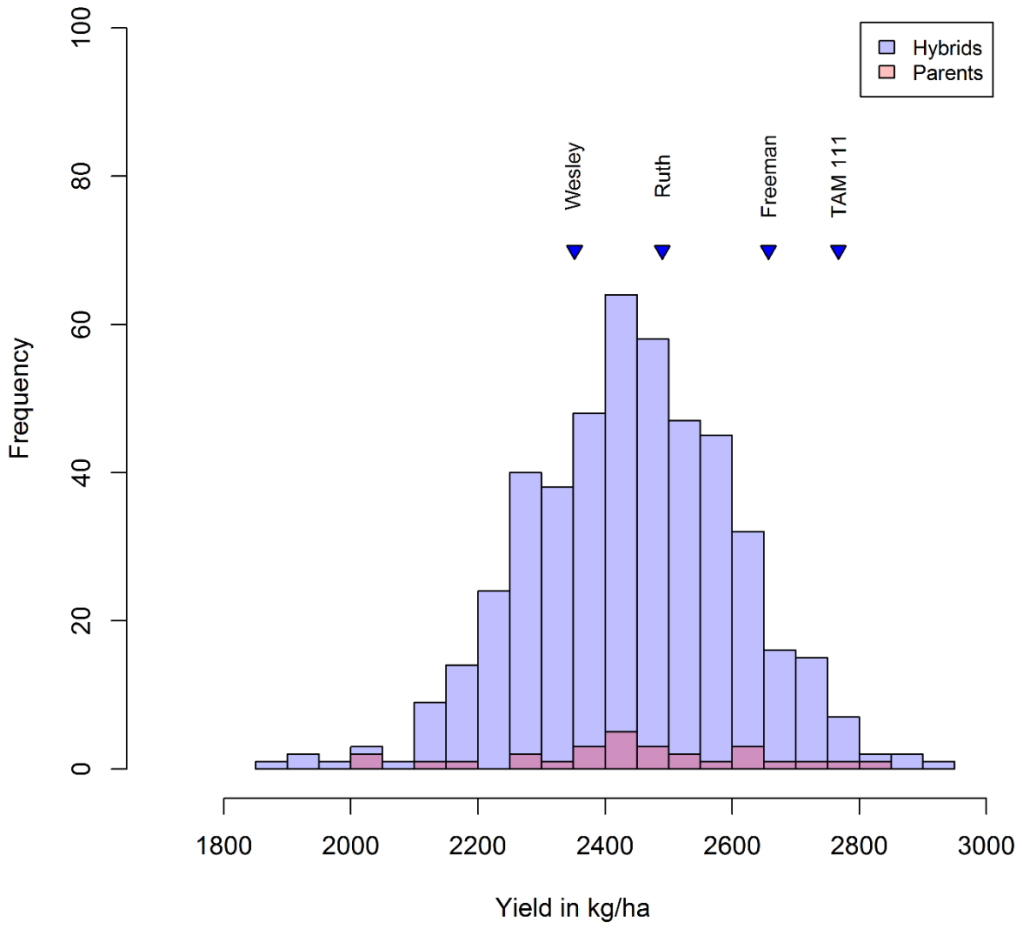

Figure 5 Grain yield of hybrids and parents in experiment 2. The yield of commercial check varieties are represented by an inverted triangle labeled with their respective names.

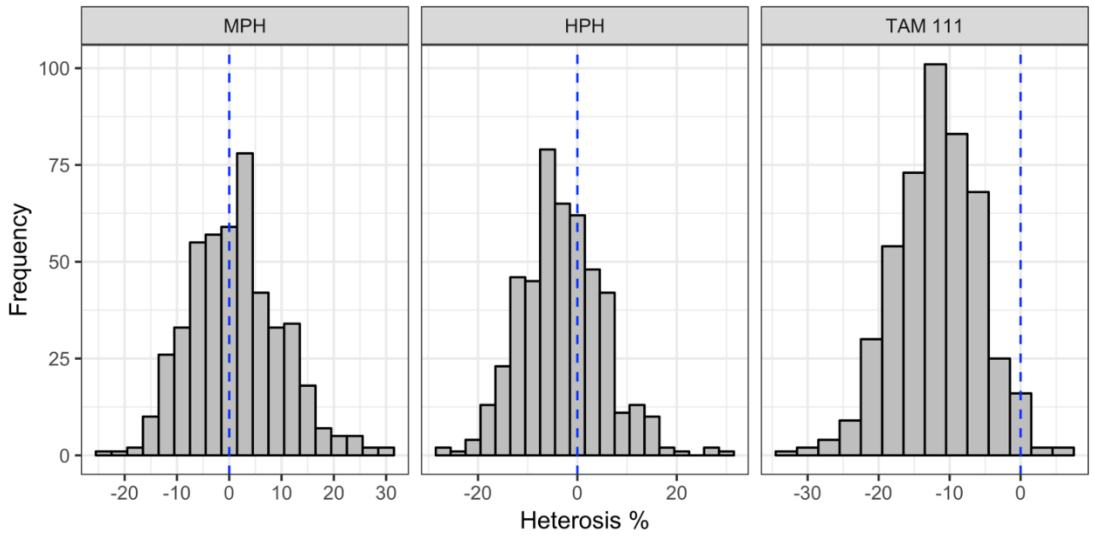

Figure 6 Mid-parent high-parent and commercial heterosis as compared to best check TAM 111, estimates from experiment 2 represented, respectively by "MPH", "HPH" and "TAM 111".

Among the three individual environments, Bushland had the highest overall mean for grain yield (See Table 2 on p. 31). Grain yield in Bushland ranged from 2065 kg ha^{-1} to 3707 kg ha^{-1}. The best performing check in Bushland was Ruth with an average yield of 3265 kg ha^{-1} and the best performing pureline was NE09517-1 from Nebraska with yield of 3428 kg ha^{-1}. The highest yielding line in Bushland was also the same hybrid NXB15-7186 with a yield of 3707 kg ha^{-1}. Only four hybrids yielded higher than NE09517-1, whereas 18 lines yielded higher than the best check Ruth. The grain yield in Greenville, which was the lower performance environment of the three environments, ranged from 1238 kg ha-1 to 2673 kg ha^{-1}. The highest yielding line in Greenville was a TX × NE hybrid (NXB15-6006, TX11D3049 × NE07531) with yield 2673 kg ha^{-1} whereas the best performing pureline was a NE parent Harry. Only one line had higher yield than Harry in Greenville whereas 10 hybrids yielded higher than the best check Freeman which had a yield of 2478 kg ha^{-1}. High parent heterosis estimates in Greenville ranged from –49.33 to 77.32 % whereas in Bushland it ranged from –27.98 to 31.53% (See Table 2 on p. 31).

Commercial heterosis estimates in Greenville ranged from –50.06 to 7.86% whereas in Bushland it ranged from –36.73 to 13.53% (See Table 2 on p. 31).

2.3.6 Reciprocal effects

A one-way ANOVA of two groups of hybrids separated by crosses and reciprocal crosses in both experiments found significant differences between them (Figure A2). A paired t-test was conducted as a follow-up of these results, which revealed that a total of 48 out of 271 in 2016 and 43 out of 271 in 2017 parental cross combinations has significant reciprocal differences (Table A2).

2.3.7 General and specific combining abilities

Both σ^2 GCA and reciprocal σ^2 GCA were significantly different from zero (Table 3). The σ^2 SCA and reciprocal σ^2 SCA were not significantly different from zero (Table 3). Presence of a high additive genetic variance was observed along with the absence of dominance genetic variances. The narrow sense heritability calculated based on the additive and genetic variance estimates was 0.51. There were no pre-determined male and female lines in the germplasm used for this study. Hence, a full diallel mating design was used for producing the F_1 seed, which means that all the lines were used as both males and females in the crossing block.

Table 3 Variance components, their corresponding variance estimates and standard errors from the diallel analysis with trait grain yield in a combined dataset with all hybrids included, Texas by Nebraska and Nebraska by Texas hybrids only, Texas by Texas hybrids only and Nebraska by Nebraska hybrids only, respectively.

Variances components	**Combined**		**TX × NE and NE × TX**		**TX × TX only**		**NE × NE only**	
	Estimate	**SE**	**Estimate**	**SE**	**Estimate**	**SE**	**Estimate**	**SE**
$\boldsymbol{\sigma}^2$GCA	22272.31*	6668.78	20197.80*	6412.00	8035.06	4439.26	5772.66*	2281.68
$\boldsymbol{\sigma}^2\text{GCA}_{\text{reciprocal}}$	5020.73*	1743.31	8868.70*	3236.75	663.41	1036.58	23.66	394.40
$\boldsymbol{\sigma}^2$SCA	0.01	0.00	0.01	0.00	0.010	0.000	0.013	0.000
$\boldsymbol{\sigma}^2\text{SCA}_{\text{reciprocal}}$	447.68	1543.73	0.01	0.00	0.010	0.000	0.006	0.000
$\boldsymbol{\sigma}^2$Error	119387.00	4747.00	105706.00	5795.00	97621.00	8474.00	56949.00	4022.00
Phenotypic	174420.00	14383.01	163840.00	15247.00	115020.00	11990.00	172450.00	45774.00
Additive	89089.00	26687.01	35475.00	12943.00	32140.00	17784.00	23091.00	91303.00
Dominance	0.05	0.01	0.04	0.01	0.04	0.01	0.05	0.01
[§]Heritability	0.51	0.12	0.22	0.07	0.28	0.14	0.14	0.02

*Significantly different from zero, $P \leq 0.05$
[§]Narrow sense heritability
** Significant at $P \leq 0.001$
[§]Not significant.

Table 4 General combining abilities (GCA) estimates of wheat inbred lines from University of Nebraska and Texas A&M University with their corresponding standard errors from a diallel mating design using grain yield data.

Genotype	Origin	Female	SE	Male	SE
FREEMAN	NE[a]	4.09	41.72	-3.898	28.46
GOODSTREAK	NE	-141.9	42.88	22.77	29.97
HARRY	NE	-158.6	47.86	-31.22	34.6
LCH13NEDH-11-24	NE	-266.5	52.12	161.4	38.62
NE07531	NE	-165.1	42.43	86.84	29.4
NE09517-1	NE	61.03	45.77	-3.991	33.02
NE10478-1	NE	264.1	85.63	59.55	65.28
NE10589	NE	-37.83	42.14	3.565	28.97
NE10683	NE	-172.8	41.79	69.47	28.55
OVERLAND	NE	-54.95	42.47	2.434	29.41
PANHANDLE	NE	-120.1	42.37	10.24	29.31
PSB13NEDH-15-58W	NE	-91.9	41.91	42.28	29.46
ROBIDOUX	NE	-95.84	42.34	5.097	29.26
SETTLER CL	NE	-167.8	46.63	57.05	34.03
WESLEY	NE	-122	42.04	-94.4	29
TX09D1172	TX[b]	-23.54	42.73	21.76	29.7
TX10D2063	TX	102.1	45.38	47.61	32.69
TX10D2230	TX	69.88	41.28	34.67	27.85
TX10D2363	TX	187.8	45.77	47.73	33.12
TX11D3008	TX	13.4	45.89	86.48	33.19
TX11D3026	TX	83.65	46.29	105.9	33.61
TX11D3049	TX	-1.224	49.18	72.32	36.44
TX11D3112	TX	107.4	41.84	70.14	28.69
TX11D3129	TX	96.51	41.94	15.51	28.86
TX12M4004	TX	166.5	44.55	48.22	31.9
TX12M4063	TX	268.9	42.09	102.5	29.14
TX12M4065	TX	194.7	41.61	20.03	28.56

[a]Inbred lines from wheat breeding program at University of Nebraska, Lincoln
[b]Inbred lines from wheat breeding program at Texas A&M University

The GCA estimates ranged from 268.90 ± 42.09 kg ha^{-1} (TX parent, TX12M4063) to -266.50 ± 52.12 kg ha^{-1} for (NE parent, LCH13NEDH-11-24) (Table 4). Seventeen out of 27 lines (ten from NE and seven from TX), had a GCA estimate significantly different from zero ($P < 0.05$). Only one out of ten lines from NE (NE10478-1) had a positive significant GCA (264.10 ± 85.63 kg ha^{-1}) while all seven TX lines had a positive significant GCA ranging from 96.51 ± 41.94 kg ha^{-1} to 268.90 ± 42.09 kg ha^{-1}. Eight out of 27 lines had a significant reciprocal GCA estimate (Table 3). Four lines each from TX and NE had a significant reciprocal GCA. Seven of the eight lines had a significant positive reciprocal GCA ranging from 69.47 ± 28.55 kg ha^{-1} for NE10683 to 161.40 ± 38.62 kg ha^{-1} for LCH13NEDH-11-24. Only Wesley had a significant negative reciprocal GCA (-94.40 ± 29.00 kg ha^{-1}).

In addition to a combining ability analysis in the full dataset, three separate analyses were conducted in the three hybrid datasets, subsetted according to the origin of the parents. In the mixed subset (TX x NE and NE x TX), σ^2 GCA and reciprocal σ^2 GCA were significantly different from zero while the σ^2 SCA and reciprocal σ^2 SCA were not (Table 3). In the TX and NE datasets, only σ^2 GCA was significantly different from zero. σ^2 GCA was three to four times higher in mixed dataset as compared to TX and NE datasets. Individual parent GCA estimates were not much different in mixed vs TX datasets for TX parents. For NE parents, the GCA estimates were higher in mixed dataset as compared to NE dataset (Figure A3). Highest GCA estimates for TX parents were observed in the full dataset, whereas for NE parents highest GCA estimates were observed in mixed dataset. However, in general GCA estimates for NE parents were mostly negative across all analyses (Figure A3). This effect is visible when the yield of the three groups of hybrids are compared. The average yield of the TX x TX hybrid group is the

highest followed by the TX x NE and NE x NE (Figure A4). However, the highest yield on individual hybrid level is observed in crosses involving parents from NE and TX (Figure A4).

2.3.8 Path coefficient analysis of yield components

There was a weak positive correlation between number of productive tillers and grain yield in hybrids whereas the correlation was negative in case of purelines (Table 5). Significant correlations were observed between each of seed per spike and thousand kernel weight with grain yield in both hybrids and purelines. The correlation coefficients for purelines were much higher than hybrids. There was a positive correlation between biomass and yield in both hybrids and purelines, but it was significant only in purelines.

The most differences in direct effects of yield components on grain yield in between hybrids and purelines was observed in number of productive tillers (Table 5). The number of productive tillers had a positive direct effect on grain yield in case of hybrids whereas it has a negative direct effect in purelines. There were strong direct effects of seeds per spike and seed weight on grain yield in both hybrids and purelines (Table 5). The direct effect of biomass on grain yield was negative in case of hybrids whereas it was weak in case of purelines. The effect of biomass on yield was manifested via strong indirect effects via seeds per spike and seed weight.

Table 5 Direct effects, indirect effects and total correlation with grain yield of yield components of hybrid and pureline wheat in yield trial at Greenville, Texas in 2017.

	‡Tillers	Seeds per spike	†Seed weight	Biomass	Correlation
			Hybrids		
‡Tillers	**0.18**	-0.06	-0.03	-0.01	0.08
Seeds per spike	-0.03	**0.35**	0.03	-0.01	0.34*
†Seed weight	-0.01	0.02	**0.40**	0.00	0.41**
Biomass	0.07	0.13	0.07	**-0.01**	0.25
			Purelines		
‡Tillers	**-0.15**	-0.05	-0.09	0.05	-0.24
Seeds per spike	0.02	**0.49**	0.12	0.06	0.69**
†Seed weight	0.04	0.16	**0.37**	0.05	0.63**
Biomass	-0.07	0.25	0.17	**0.11**	0.46**

*Significance at the 0.05 probability level.
**Significance at the 0.01 probability level.
†Thousand kernel weight.
‡Number of effective tillers per plot or tillers that had heads.

2.4 Discussion

2.4.1 Efficacy of chemical hybridizing agent and its carryover effect on grain yield

Due to the unreliability of CMS based hybrid seed production in wheat, CHAs are most commonly used for hybrid seed production (Whitford et al. 2013). The advantages of using CHAs over CMS based methods is the ease of converting any potential parental line into female and estimating its GCA and full restoration of fertility in hybrids (Cisar and Cooper, 2002). Due to these advantages, CHAs were extensively used in the past and are currently being used in hybrid wheat research (Basnet et al. 2019). Some of the CHA's that were developed in the past had negative impact on grain yield when they were used for hybrid seed production (Adugna et al., 2004; Whitford et al., 2013; Wong et al., 1995). A very important consideration for its use in the hybrid wheat breeding programs of UNL and TAMU was its effectiveness in producing

hybrid seed. Based on the data from this study, we can conclude that CHAs are very effective in producing hybrid seed in the US Great Plains conditions.

Another important consideration before CHA use, is its potential negative impact on seed quality, hence on hybrid physiology, growth and ultimately grain yield under testing conditions. A one-way ANOVA of groups of lines selfed naturally and selfed via use of CHA revealed that there is no significant difference in yield (data not shown). This result led us to conclude that CHA did not have a significant impact on grain yield of hybrids. This result was expected as the CHA used for hybrid seed production was provided by Saaten Union Recherche (now Asur Plant Breeding) and has been successfully used for commercial European hybrid wheat seed production since the early 1990s (https://www.saaten-union.fr/).

2.4.2 Reciprocal effects

The reciprocal effect was significant only in ~ 15% of crosses. This implies that in general, any lines could be used as males or females in hybrid seed production. In addition, there seems to be evidence for maternal effects, as evidenced by $\boldsymbol{\sigma}^2\text{GCA}_{\text{reciprocal}}$ estimates in the diallel analysis. Variance associated with reciprocal GCA effects are due to maternal effects and cytoplasmic genetic effects that are shared by all hybrids with a common female parent, but not shared by crosses where that parent is used as male (Isik et al., 2017). $\boldsymbol{\sigma}^2\text{GCA}_{\text{reciprocal}}$ was only significantly different from zero in the full dataset and the mixed dataset only but not significantly different from zero in TX dataset and NE dataset. This implies that reciprocal effects are important only when crosses are made with parents from both NE and TX. Maternal effects in reciprocal crosses in wheat have been reported in several previous studies (Millet et al 1983; Millet et al 1991) and has been attributed to the higher amount of contribution of female parent to the triploid endosperm (2n) (Bingham 1966; Wegel and Shaw 2005).

Because CHA was used for seed production, some of the hybrids may have included selfed seed or outcrosses due to difference in flowering time between the male and female parents. This could lead to significant reciprocal effects. For example, if a female parent has a higher yield than a male parent in a cross, the cross hybrid would have a higher yield as compared to a reciprocal cross hybrid in presence of selfing. When there is selfing during crossing, the cross hybrid seed will have a contribution of the higher yielding female parent seed as compared to the reciprocal cross hybrid which will have a contribution of the lower yielding male parent seed.

2.4.3 Hybrid wheat grain yield and heterosis

Grain yield heterosis in wheat has been the interest of researchers as early as 1935 (Pal and Alam, 1938). The interest has ebbed and flowed into the 1960-1970 period (Briggle, 1963; Hermsen, 1960; Johnson and Schmidt, 1968; Knott, 1965) and the 1990s to 2000s (Barbosa-Neto et al., 1996; Borghi and Perenzin, 1994; Dreisigacker et al., 2005; Shamsuddin, 1985; Uddin et al., 1992) according to commodity prices and investment in hybrid wheat research (Pickett, 1993; Virmani and Edwards, 1983). Most of these earlier studies were conducted using small number of hybrids, and sometimes growing hybrids in hill plots because of the difficulty in producing seed. Thus, earlier experiments likely had inflated, or imprecise estimates of heterosis (Dreisigacker et al., 2005). More recent studies were conducted using higher number of hybrids and in yield plots, and provide more precise estimates of heterosis in hybrid wheat (Barbosa-Neto et al., 1996; Dreisigacker et al., 2005). More recent ones (Basnet et al., 2019; Gowda et al., 2010; Gowda et al., 2012; Zhao et al., 2015) in which hybrid seed was produced using CHA's have larger sample sizes, more precise estimates of grain yield heterosis and also represent the current methods and practices of growing wheat.

Most of the early studies of heterosis in hybrid wheat studied only mid-parent heterosis (Pal and Alam, 1938; Shamsuddin, 1985). Knott (1965) reported presence of high-parent heterosis but no commercial heterosis in the seven hybrid genotypes that he evaluated in comparison to 'Thatcher'. Uddin et al. (1992) studied all three types of heterosis; mid-parent, high-parent and commercial heterosis in Australian spring wheat but the experiment was conducted in hill plots and the heterosis estimates are most likely inflated. The study by Barbosa-Neto et al. (1996) was one of the reliable early hybrid wheat studies since they evaluated 722 soft red winter wheat hybrids in multiple years and locations in experimental yield plots rather than hill plots. They reported mid-parent heterosis in the range of -20 to 57% and high-parent heterosis in the range of -22 to 47% but commercial heterosis was not studied or reported. The parents for these hybrids were randomly selected, hence the heterosis estimates might be lower than in studies where some consideration is given to select genetically dissimilar parents to exploit heterosis. Dreisigacker et al. (2005) examined heterosis in a set of 112 hybrids developed from widely successful CIMMYT wheat varieties and some Chinese lines over two years and reported -15.33 to 14.33 % mid-parent heterosis whereas no positive high parent heterosis and commercial heterosis was observed. Liu et al. (1999) also reported low mid-parent heterosis in Chinese spring wheat from an experiment evaluating 41 hybrids. Dreisigacker et al. (2005), based on their results, concluded that research investment is not justified in hybrid wheat because of the low amount of heterosis in hybrid wheat. However, the hybrid wheat research resumed in CIMMYT almost a decade after that in 2012 with promising results (Basnet et al., 2019). In a most recent publication from CIMMYT hybrid wheat breeding program involving 1888 experimental hybrids and 685 parents, grain yield heterosis was on average 0.43 to 0.68 t ha^{-1} or 7.5 to 9.5%, which is very promising.

More recent studies, have reported higher estimates of heterosis (Gowda et al., 2010; Gowda et al., 2012; Zhao et al., 2015). Zhao et al. (2015) produced 1604 single cross hybrids using 135 diverse winter wheat lines adapted to Central Europe and evaluated them in 11 field trials. In their study, a total of 97 hybrids outperformed the best commercial check 'Tobak' with a yield advantage as high as 1 t ha^{-1} (Zhao et al., 2015). Similarly, Gowda et al. (2012) evaluated 940 winter wheat hybrids developed by the French hybrid cereal breeding company Saaten-Union in four experiments in France and reported positive commercial heterosis of about 4-5% in two out of four experiments. The experimental hybrids evaluated in that experiment came from crossing 334 female lines and 114 male lines in incomplete factorial crosses. More than 150 lines evaluated across four experiments performed better than the best commercial check in respective experiment. The best hybrid yielded 1 t ha^{-1} higher than the best commercial check in two of the experiments. In durum wheat (*Triticum turgidum* ssp. *durum*) also hybrids have been shown to out yield best checks by more than 1 t ha^{-1} or 22 % (Gowda et al., 2010).

In this study, high-parent heterosis ranged from -29% to 77% in the three trials. The 77% high-parent heterosis came from the trial planted in Greenville, which had presence of some disease pressure in 2017. The disease infection might have affected the performance of parental lines and ultimately the heterosis estimates. In a commercial hybrid breeding program for self-pollinated crops, it is best to look at commercial heterosis (% increase in grain yield over the best commercial check) rather than mid-parent or high-parent heterosis. Commercial heterosis in this study was also higher than most of the older hybrid wheat studies discussed above and more in line with the studies done in winter wheat in Europe. The best commercial

heterosis estimate came from experiment 1 in McGregor with ~ 21%, followed by 13.5% in Bushland and ~ 6-7 % in Greenville and combined analysis.

Most of the older studies had very small sample sizes and parents used probably came from the same line breeding programs and hence were very similar. In our study, the number of parents used was higher than most of the older studies. In addition, the parents used in this study were best performing lines from two different line breeding programs in Nebraska and Texas. Nebraska and Texas have very different growing conditions with the planting and harvesting dates differing by more than a month. The parental lines from Texas came from two breeding stations in College Station in south Texas and Amarillo in Northwest Texas, which serve different geographical locations and growing conditions. Most likely due to these differences in locations, growing conditions and germplasm, the heterosis estimates are promising in this study.

Angus (1997) estimated that a commercial heterosis in comparison with the best line bred variety of about 5% is needed for hybrid wheat to become economically viable. Similarly, (Pickett and Galwey, 1997) concluded that 6-34% of mid-parent heterosis is needed for the commercial success of hybrid wheat. These numbers might now be a little higher considering these estimates were made decades ago, but based on these benchmarks, we can conclude that heterosis in our current hybrid wheat breeding program is enough to show commercial hybrids to be viable.

2.4.4 General and specific combining abilities and their implications in hybrid wheat breeding

Numerous studies calculating the GCA and SCA in wheat have been previously done to guide selection of parents and identification of heterotic pattern in wheat (Basnet et al., 2019;

Borghi and Perenzin, 1994; Corbellini et al., 2002; Dreisigacker et al., 2005; Gowda et al., 2010; Gowda et al., 2012) and also in other cereal crops where hybrid breeding efforts are ongoing (Oettler et al., 2003; Fischer et al., 2010; Bernhard et al., 2017). In wheat, Dreisigacker et al. (2005) reported significant σ^2GCA and σ^2SCA and also a higher σ^2GCA to σ^2SCA ratio in CIMMYT germplasm. A similar trend was observed by Basnet et al. (2019) in a much larger study a decade later. Similarly, Gowda et al. (2012) observed a significant σ^2GCA and σ^2SCA and a higher σ^2GCA to σ^2SCA ratio. They found σ^2SCA to be significant in three out of four experiments and concluded that a significant σ^2SCA might have been a manifestation of the use of similar parents. In this study, σ^2 GCA was significantly different from zero whereas σ^2SCA was not. This clearly underscores the importance of selection of parents based on GCA effects. This also highlights the potential for improvement of hybrid performance via use of reciprocal recurrent selection. Genetically divergent groups lead to a high ratio of σ^2GCA by σ^2SCA (Fischer et al., 2010). This was evident in this study as well, when the full dataset was partitioned into three subsets and σ^2G/SCA was estimated. Based on σ^2GCA by σ^2SCA ratio in this study, we can say that the lines used for parents in this study were not genetically similar, since they were selected from two diverse winter wheat growing regions of the great plains and σ^2GCA produced due to this process can be exploited in producing high performing hybrids. There is evidence of maternal effects in this study, due to the presence of $\sigma^2GCA_{reciprocal}$ (Table 3, Table 4) which indicates that selection for female parents is going to be important in producing good hybrids. Since top cross seed has to be produced in isolation plots for every male separately, GCA tests for male lines are time and resource demanding (Gowda et al., 2012). Presence of strong maternal effects makes the selection of parents for hybrid testing easier, since more

emphasis needs to be given to selection of female parents and less emphasis to male parents for combining ability purposes.

General combining ability estimates and mid-parent values have been previously reported as tools to predict hybrid performance (Dreisigacker et al., 2005; Gowda et al., 2012). Gowda et al. (2012) reported very high correlation between sum of GCA of parents and hybrid performance while Dreisigacker et al. (2005) reported very high correlation between mid-parent value and hybrid performance. We tested the correlation between mid-parent value and sum of GCA of parents with hybrid performance and found GCA to be a better predictor of hybrid performance than mid-parent value (Figure 7).

2.4.5 Path coefficient analysis

Path coefficient analysis is a standardized partial regression coefficient that estimates the direct influence of independent variables on to dependent variables and permits the separation of correlation coefficients into components of direct and indirect effects while reducing confounding effects of multicollinearity (Akanda and Mundt, 1996; Dewey and Lu, 1959). Path coefficient analysis has been used in wheat breeding and physiology to study the true nature of cause and effect relationship of yield components with grain yield and identify true genetic associations between traits as compared to correlation only analysis, on a phenotypic level (Bhatt, 1973; Cooper et al., 2012; Garcia del Moral et al., 1991; Simane et al., 1993). The main objectives of this analysis were to conduct a yield component analysis in hybrid wheat, which has never been reported before and also to identify differences between relationship of yield and yield components in hybrids and pureline wheat. In hybrid wheat we observed that seed weight has the highest direct effect on grain yield followed by seeds per spike and number of tillers whereas in purelines it was seed per spike followed by seed weight (Table 5).

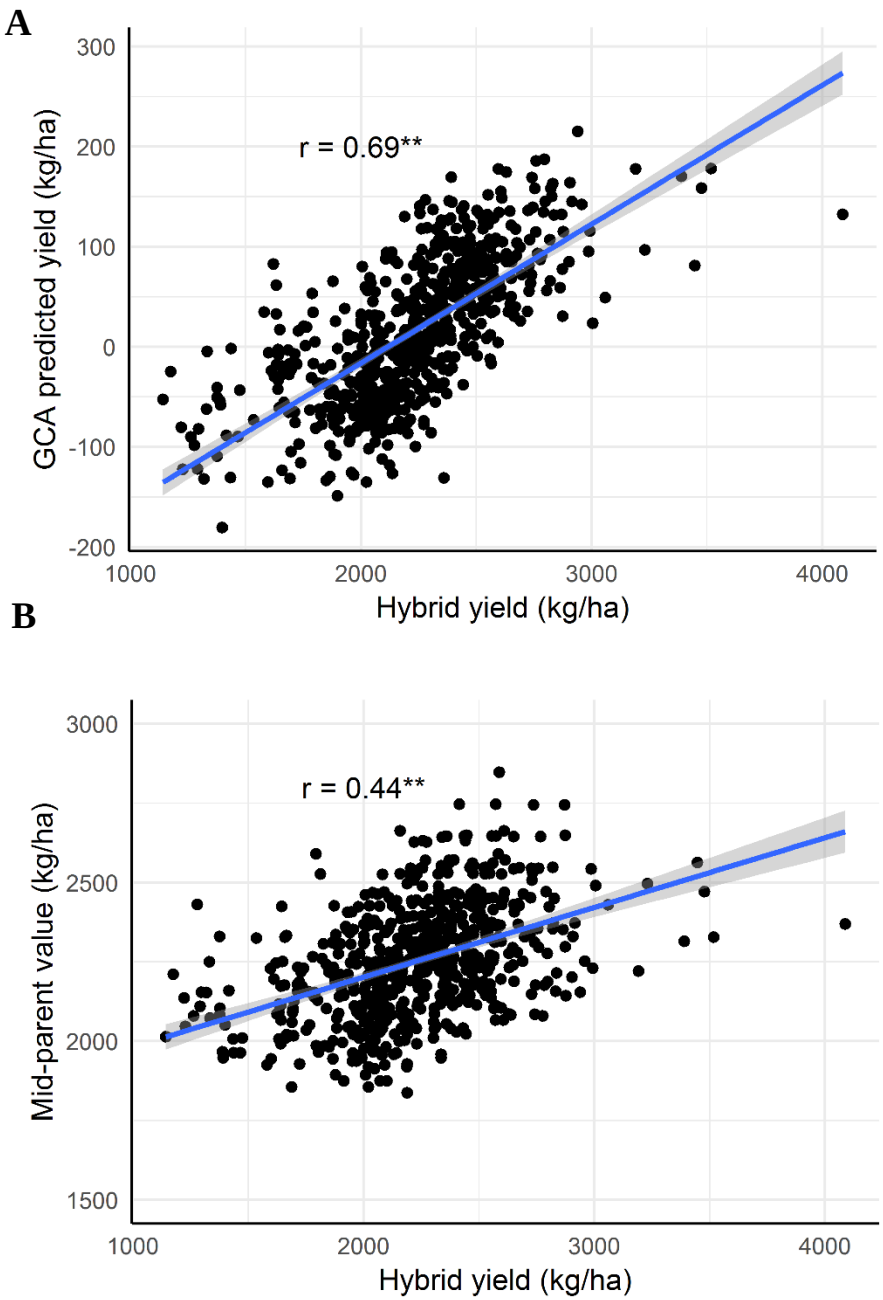

Figure 7 Correlation between grain yield of hybrid performance and (A) general combining ability (GCA) and (B) mid-parent value. **Significant at the 0.001 probability level.

This result is not surprising considering that hard wheat breeders have been selecting against seed weight and favor seed per spike to maintain the high protein content that is favored by bakers and millers (Griffiths et al., 2015). The most important takeaway from this analysis was that the major difference in yield components between hybrid wheat and pureline wheat lies in the effect of tillering capacity on grain yield. Number of productive tillers had a positive direct effect on grain yield in hybrid wheat whereas it had negative effect in pureline wheat. This could be an indication that the grain yield heterosis is a result of differences in tillering capacity between pureline and hybrid wheat.

The correlation between productive tillers and grain yield was low whereas correlation between biomass and grain yield was high in hybrids. After partitioning the correlation coefficient into direct and indirect effects only, the high direct effect of tillering on grain yield and low direct effect of biomass was visible. This proves the utility of path co-efficient analysis in identifying true associations. Making conclusions about relationship of yield components based on observation of correlations only can sometimes be erroneous.

2.5 Conclusions

Hybrid wheat offers great promise in terms of grain yield for the growers in Texas based on the data presented in this study. There is sufficient high-parent and commercial heterosis for hybrid wheat to be commercially viable in Texas based on estimates by Angus (1997) and Pickett et al. (1997). Moreover, genetic gains can be made for heterosis by breeding efforts since majority of heterosis is a result of additive gene action and use of elite lines from two breeding programs serving different geographical locations and growing conditions. On a phenotypic level, grain yield heterosis could be a result of higher tillering capacity of hybrids over pureline varieties, although data from more locations/experiments would be needed to state this

conclusively. However, this result addresses the concern that maybe higher grain yield comes at an expense of protein quality since HRWW from the US Great Plains can be discounted for lower protein content. Use of CHAs for hybrid seed production is a reliable method since it does not impact on grain yield of the resultant hybrids. CHAs can be used for hybrid seed production routinely in breeding programs for estimation of GCA until the best females are identified to be converted into CMS. GCA can be very good predictor of hybrid yield. Selection of parents for making single crosses can be made by use of GCA estimates since GCA predicts hybrid yield in wheat with high accuracy. Absence of σ^2SCA indicates that development of heterotic pools is crucial for exploitation of dominance effects in hybridization.

CHAPTER III

SUPPLEMENTING SELECTION DECISIONS IN A HYBRID WHEAT BREEDING PROGRAM BY USING F_2 YIELD AS A PROXY OF F_1 PERFORMANCE

3.1 Introduction

Producing enough hybrid seed for testing in replicated trials and multiple environments is a common problem in many self-pollinated crop species (Cox and Murphy, 1990; Ghaderi et al., 1984; Meredith Jr and Brown, 1998; Meredith, 1990; Wu et al., 2004). The problem is even more magnified when breeding programs are trying to test large number of cross combinations during the early stages of hybrid breeding pipeline. Once the F_1 hybrids are tested in limited locations/reps, copious amount of F_2 seed is produced which can be used to test hybrid genotypes in multiple environments and locations. In order to increase testing power in self-pollinated crops where seed production is limited or expensive, many researchers have resorted to using F_2 seed for testing hybrid genotypes in multi-environment trials (METs). This method has been extensively used in the past in several crop species such as such as wheat (Cox and Murphy, 1990; Winzeler et al., 1993), barley (Hockett et al., 1993), flax (Shehata and Comstock, 1971), soybeans (Friedrichs et al., 2016; Gizlice et al., 1993; Lewers et al., 1998), cotton (Meredith Jr and Brown, 1998; Meredith, 1990; Tang et al., 1993; Wu et al., 2004), triticale (Oettler et al., 2001) etc. The theoretical expectation is that heterosis observed in F_1 should decline by 50% in F_2 generation, however most of the times actual heterosis decline highly deviates from theoretical expectation. Hence, superior genotypes in F_2 populations expressing heterosis should predict and have better performance in F_1 combination. The evaluation of F_2's is a viable and promising alternative to using F_1's when hybrid seed production is limited. This F_2 method might

allow researchers to better estimate heterosis since the F_2 hybrid can be tested in multiple environments with replications.

The hybrid wheat breeding program at Texas A&M has been using chemical hybridizing agents (CHAs) for producing hybrid seed (Adhikari et al. 2020; Easterly et al. 2020). Breeders do not have heterotic pools in wheat and most often, which lines to use as male or female is not well established. Due to this issue, many crosses need to be made at the early stages and most likely in small quantities. Hybrid seed produced in low quantities in the field crossing blocks, seriously limits the program's ability to test hybrids in METs. In the context of this practical issue, we decided to explore the possibility of using F_2 in METs and relate their performance with F_1 evaluation. Hence, the objectives of this study were (i) to evaluate F_2 populations with their parents in METs across Texas and Nebraska (ii) to estimate mid-parent and high-parent heterosis of F_2 populations for grain yield using performance data from METs and (iii) to compare heterosis of hybrid crosses at F_2 stage with F_1 stage between years to aid selection decisions.

3.2 Materials and methods

3.2.1 Experimental site selection

The experiment was designed to include the major winter wheat growing regions of Texas and Nebraska. The wheat testing locations in Texas are divided into four major mega environments: South Texas, Blacklands, Rolling Plains and the Panhandle region. The wheat breeding test sites in Nebraska can be divided into two major mega environments based on climatic conditions and moisture availability; Eastern Nebraska and Western Nebraska. The F_2 testing sites represented these six environments. The target environments in Texas were Bushland, Chillicothe, Greenville and McGregor, respectively for Panhandle, Rolling Plains,

Blacklands and South Texas. In Nebraska, western Nebraska was represented by Grant and eastern Nebraska was represented by Lincoln.

3.2.2 Experiment design and plant materials

The hybrid wheat experiment included development of experimental hybrids from a full diallel mating design using 25 elite hard red winter wheat parents from wheat breeding programs of University of Nebraska, Lincoln and Texas A&M University (Adhikari et al. 2020, Easterly et al. 2020). Experimental hybrid seed was produced in field crossing blocks in Texas and Nebraska in 2015 and 2016 using chemical hybridizing agent (CHA) Croisor 100 from Saaten-Union Recherche (now Asur Plant Breeding, Estrées-Saint-Denis, France). The CHA acts as a male gametocide when sprayed before flowering at stage 34 of the Zadoks scale (Zadoks et al., 1974), which is when immature spike head are about 1.5 – 1.8 cm in length. The experimental hybrids were evaluated in three field experiments in Texas in 2016 and 2017 in McGregor, Greenville and Bushland, and at Lincoln, North Platte and Alliance in NE. Using the data from Texas, a subset of these hybrids and their respective parents were selected based on their performance and used as entries in this experiment. The entries are F2 seed produced on these experimental F1 hybrids. The F2 entries comprised of hybrid crosses selected from the F1 trials with high to low grain yield and heterosis (Figure 8).

There were 23 hybrid lines and 17 purelines/parents in each experiment i.e., 2017 and 2018. The experiment in 2017 included 40 entries selected based on F1 yield at McGregor 2016. The experiment in 2018 included 40 entries selected based on F_1 yield across all three locations. There were 28 entries repeated between the 2017 and 2018 experiments of which 11 were F_2's whereas 17 were parents. The experiment was planted in four locations in Texas and two locations in Nebraska in 2017, whereas in 2018 one location in Nebraska (Grant) and one

location in Texas (Chillicothe) was lost due to severe weather conditions. Across the two experiments, there were 54 entries, of which 33 were F_2 of hybrids evaluated in 2016 - 2017 and 21 were parents of those hybrids. The experimental design was an alpha-lattice design with 40 entries, three replications and eight incomplete blocks planted on plots of ~ 3.6 m^2 size.

3.2.3 Statistical analysis

The F_1 experiment was planted in augmented design with unreplicated entries and replicated checks. Full details about estimation of F_1 hybrid performance after use of spatial correction models is given in a previous study (Adhikari et al. 2020). For the F_2 experiment, individual locations were analyzed using analysis of variance (ANOVA) in SAS 9.4 (SAS Institute, Cary NC). The analysis was conducted by PROC GLM method using this linear model:

$$Y_{ijk} = \mu + B_i + B_i{:}I_j + G_k + \varepsilon_{ijk}$$

In this linear model, Y_{ijk} plot level yield of $_k$th hybrid in $_i$th block and $_j$th incomplete block; μ is the overall mean; B_i is the effect of $_i$th block; $B_i{:}I_j$ is the effect of the $_j$th incomplete block nested within $_i$th block; G_k is the genotypic effect of entries and ε_{ijk} is the residual. All the sources of variation were considered as random except entries. Genotypic effects of entries were estimated using LSMEANS statement in SAS 9.4.

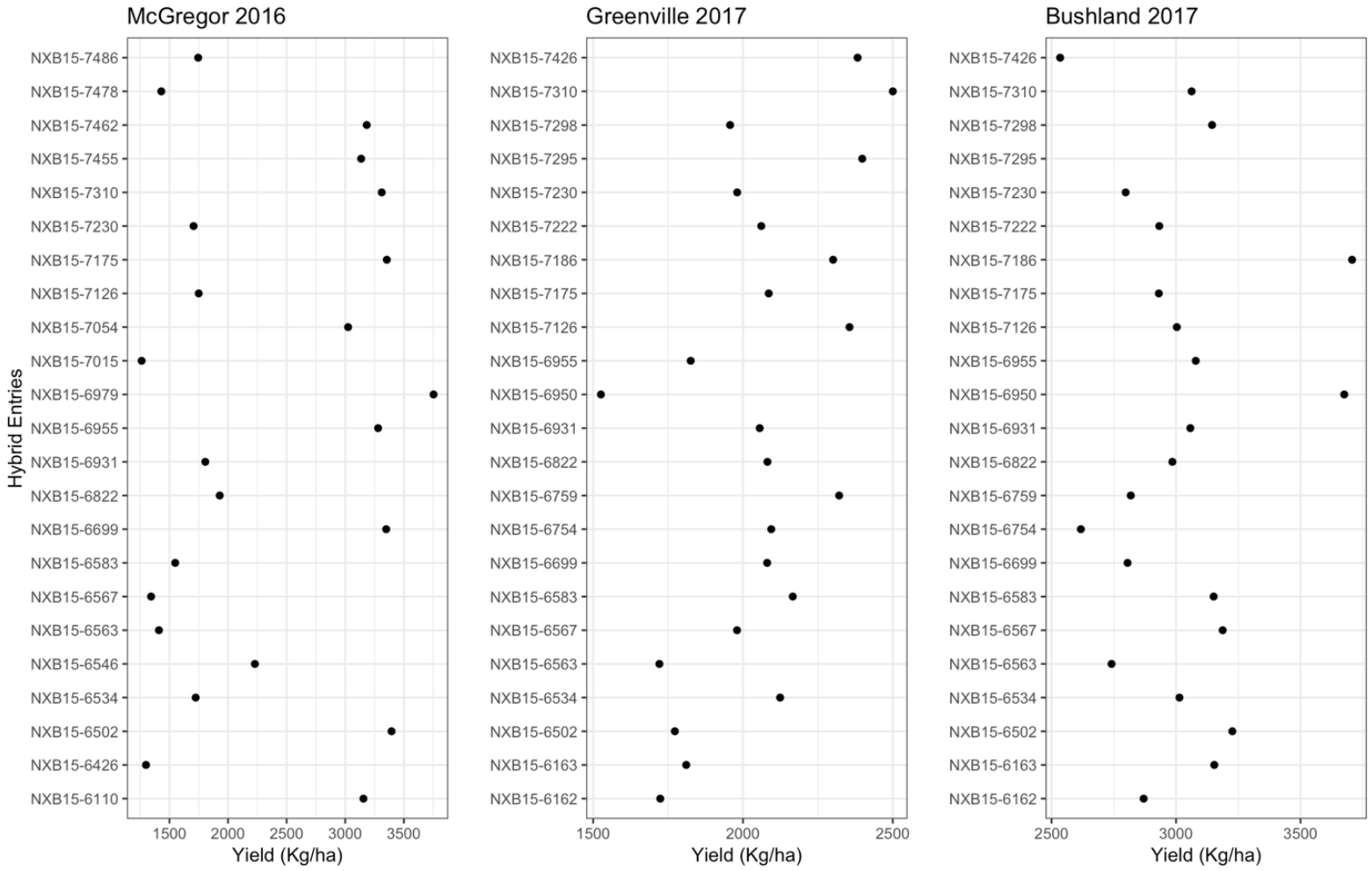

Figure 8 Performances of hybrid genotypes in F_1 experiments selected and evaluated on F_2 experiments in 2017 and 2018.

After the analysis of single environments, a combined analysis was conducted for all environments combined within each year. The combined analyses were conducted using the following linear model:

$$Y_{ijkl} = \mu + E_l + E_l\,(B_i) + E_l\,[B_i\,(I_j)] + G_k + E_l{:}G_k + \varepsilon_{ijk}$$

In this linear model Y_{ijkl} plot level yield of $_k$th hybrid in $_i$th block and $_j$th incomplete block in in $_l$th environment; μ is the overall mean; E_l is the effect of $_l$th environment; $E_l\,(B_i)$ is the effect of $_i$th block in $_l$th environment; $E_l\,[B_i\,(I_j)]$ is the effect of the $_j$th incomplete block nested within $_i$th block in $_l$th environment; G_k is the genotypic effect of entries; $E_l{:}G_k$ is the genotype by environment interaction and ε_{ijk} is the residual. All the sources of variation were considered as random except genotypes.

In addition to this combined analysis within each year, a GGE biplot analysis was conducted to group environments into homogeneous groups using 'gge' package (Wright 2018) in R version 3.5 (R core team, 2017). A combined ANOVA was conducted for these homogeneous groups based on GGE biplot analysis and genotypic effects were estimated using LSMEANS method in SAS 9.4. For the GGE biplot analysis, all the factors were treated as fixed except the residuals.

The broad sense heritability for each field trial was calculated using the formula

$$H^2 = VG/\,(VG + VE/r),$$

where VG is genetic variance, VE is error variance and r is number of replications.

3.2.4 Heterosis estimation and comparison with F_1 estimates

Heterosis of these entries in F_1 generation, has been reported for all three locations in the earlier study (Adhikari et al. 2020). Theoretically, the heterosis estimate decreases by 50% from F_1 to F_2 generation (Falconer and Mackay 1996). Since, the parents of F_2 entries were included in

all the F_2 trials, we were able to calculate mid-parent and high-parent heterosis for the entries. A comparison was made between F_1 heterosis estimated from F1 evaluation trials of 2016-2017 and F_2 heterosis. The F_2 heterosis was calculated using data generated in this study whereas the F_1 heterosis came from the earlier published work. Mid-parent heterosis (MPH) was calculated as using the formula MPH = 100 × (F2 – MPV) / MPV whereas high-parent heterosis was calculated using the formula HPH = 100 × (F2 – HPV) / HPV.

3.3 Results

3.3.1 Grain yield

Mean grain yield of test environments in 2017 ranged from 1116 Kg ha^{-1} in McGregor, Texas to 4667 kg ha^{-1} in Lincoln, Nebraska (Table 6). In Texas, test environment Bushland was the highest yielding trial with least amount of variance around the mean whereas McGregor was the environment with lowest grain yield (Figure 9). However, in McGregor test entries had a high variance for grain yield and it ranged from ~ 500 kg ha^{-1} to 2000 kg ha^{-1}. Among the two locations in Nebraska, Lincoln was the higher yielding environment as compared to Grant. The mean grain yield in the combined analysis across environment was 2586 kg ha^{-1}. The effect of genotypes was significant in all environments and genotype and environment interaction (G × E) was significant in the combined analysis (Table B1).The broad sense heritability calculated on entry mean basis ranged from 0.63 – 0.86 for single locations and 0.67 for the combined analysis. The co-efficient of variation ranged from 8.88 to 22.67 % across the six trials.

In three out of six locations (Bushland, Chillicothe and Grant), the highest yielding entry was an F_2 hybrid not a pureline (Table 6). In all three locations, the F_2 hybrid had a significantly higher yield than the highest yielding pureline (LSD, $p \leq 0.05$). In the combined analysis, a TX

parent (TX10D2230) had the highest yield which was significantly higher than the highest yielding F_2 line (Table 6).

In 2018, experiments in two of the test environments Chillicothe, Texas and Grant, Nebraska were lost due to extreme weather events. Those two experiments were not harvested and are not included in the final analysis. Among the four test environments, Lincoln had the highest mean grain yield (5343.63 Kg ha^{-1}) followed by Greenville, Texas (4631.78 Kg ha^{-1}). Bushland which was the trial with highest average yield in 2017 in Texas was the trial with lowest average yield (1717.87 Kg ha^{-1}) in 2018. McGregor, TX which was the location with lowest overall mean in 2017 (1116.26 Kg ha^{-1}) had the third highest average yield in 2018 (3697.15 Kg ha^{-1}). The average yield across all four locations was 3843.95 Kg ha^{-1}. Significant differences between genotypes was observed in all test environments and the G × E effect was significant in the combined analysis.

Table 6 Summary statistics from analysis of variance conducted for single environments and a multi environment combined analysis.

Trial	Bushland	Chillicothe	Grant	Lincoln	Greenville	McGregor	Combined
2017							
Highest yielding entry							
Line	TX12M4065	TX10D2230	Freeman	Robidoux	TX11D3008	TX12M4063	TX10D2230
Hybrid	NXB15-6110	NXB15-6955	NXB15-6534	NXB15-6546	NXB15-7462	NXB15-6110	NXB15-6110
Highest yield (Kg ha^{-1})							
Line	3635.30	2102.58	3009.18	6151.44	3543.66	2216.54	3067.03
Hybrid	3871.30	2163.01	3245.45	5560.48	3115.81	2124.83	2997.88
R^2 (%)	0.80	0.85	0.76	0.84	0.72	0.95	0.95
CV (%)	8.88	10.31	12.29	8.91	22.67	22.03	14.15
Mean (Kg ha^{-1})	3094.54	1724.92	2610.66	4667.25	2306.89	1116.26	2586.76
LSD (Kg ha^{-1})	50.76	32.82	59.23	76.83	96.54	45.41	27.60
H^2	0.79	0.78	0.63	0.83	0.68	0.86	0.67
2018							
Highest yielding entry							
Pureline	Wesley	-	-	TX10D2363	TX10D2363	Overland	TX10D2363
hybrid	NXB15-6583	-	-	NXB15-7186	NXB15-6567	NXB15-7186	NXB15-7186
Highest yield (Kg ha^{-1})							
Pureline	2102.13	-	-	6252.01	5438.69	4254.10	4296.38
Hybrid	2169.16	-	-	6231.83	5594.09	4498.96	4300.86
R^2 (%)	0.83	-	-	0.86	0.74	0.55	0.94
CV (%)	13.07	-	-	5.19	8.48	17.14	11.76
Mean (Kg ha^{-1})	1717.87	-	-	5343.63	4631.78	3697.15	3843.95
LSD (Kg ha^{-1})	45.41	-	-	51.20	72.52	117.04	41.74
H^2	0.67	-	-	0.81	0.64	0.73	0.55

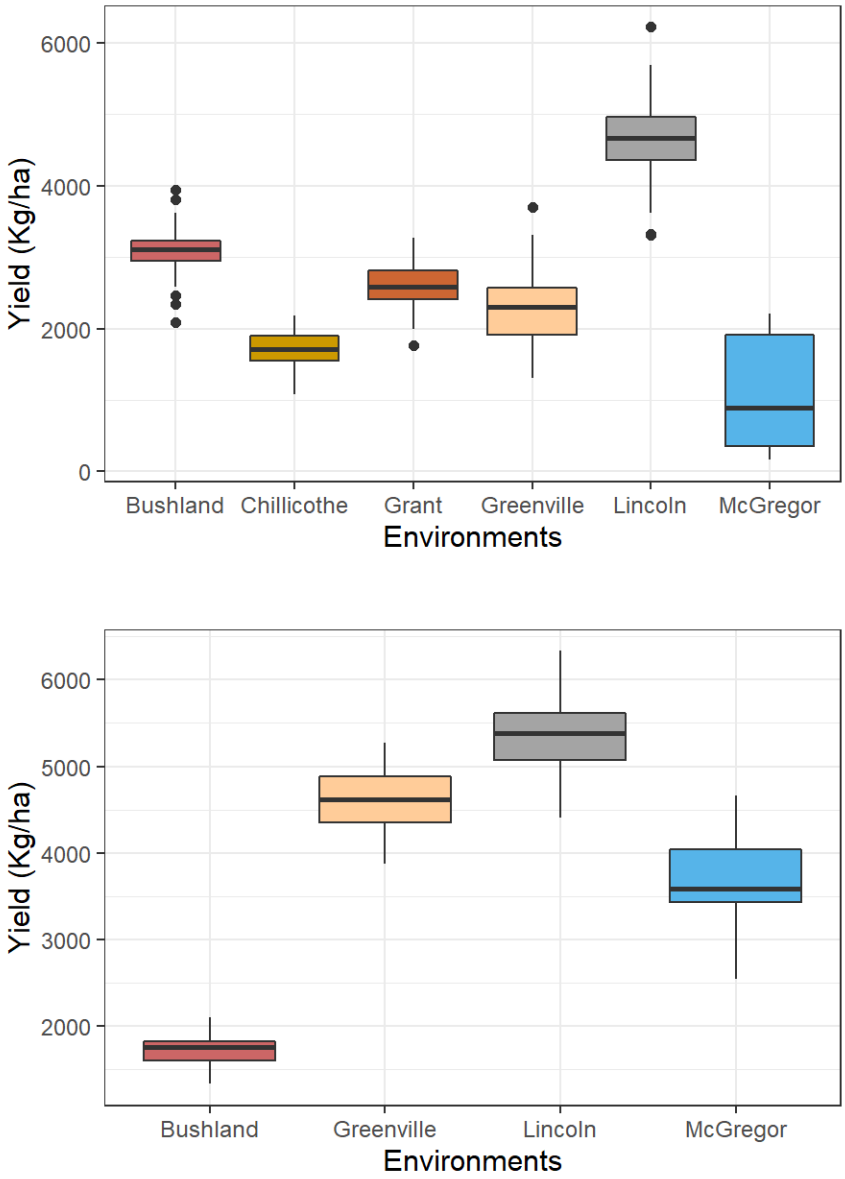

Figure 9 Boxplots representing performance of F_2 hybrids in different environments in A) 2017 and B) 2018.

In all three locations from TX in 2018, the highest yielding entry was a F_2 hybrid entry (Table 6). In these three trials, the highest yielding hybrid had a significantly higher yield than the highest yielding pureline. In Lincoln, a TX parent line TX10D2363 was the highest yielding entry but its yield was not significantly higher than the highest yielding hybrid (Table 6). A GGE biplot analysis of the G × E allowed us to group environments into homogeneous groups. AN environment-vector view of the GGE biplots for two experimental years 2017 and 2018 are given in Figure 10.

According to Yan and Tinker 2006, an acute angle between the environment vectors in biplot represents similar environments for their ability of discriminating genotypes tested in them. An acute angle between the environment vectors in GGE biplot indicates, low amount of crossover interaction in G × E whereas an obtuse angle between environment vectors represents presence of high amount of crossover G × E. Based on that, in 2017, two groups of environments were constructed. The first group consisted of three locations in Texas i.e., Bushland, Chillicothe and McGregor (Fig. 3.3) while the second group consisted of two locations in Nebraska i.e., Grant and Lincoln. A comparison between the groups and combined analysis across all locations revealed a decrease in G × E mean squares. However, the G × E effect was still significant in the two groups constructed. Similarly, in 2018 a GGE biplot analysis revealed that Lincoln, Nebraska and McGregor, Texas were the two similar environments. Hence, a combined ANOVA of these two locations was constructed. The G × E mean squares was not reduced in this group as compared to the combined analysis of all four locations (Table B1).

3.3.2 Comparison between F_1 and F_2 heterosis

Heterosis was observed in F_2 populations in both experiments in 2017 and 2018. Heterosis in F2 populations were observed in single locations as well as combined analyses across locations. In 2017, the mid-parent heterosis estimates ranged from -20.97 to 27.52 % whereas the high-parent heterosis estimates ranged from -21.07 to 17.85% in combined analysis across six locations (Figure 11). In F_1 stage, the mid-parent heterosis estimates ranged from -76.52 to 36.86% whereas the high-parent heterosis estimates ranged from -93.54 to 34.89% (Table B4). In 2018, the mid-parent heterosis estimates of F_2 populations ranged from -9.9 to 9.17% whereas the high-parent heterosis estimates ranged from -16.57 to 6.68% in combined analysis across four locations (Figure 11). In F_1 stage, the mid-parent heterosis of these crosses ranged from -16.06 to 23.86% whereas the high-parent heterosis estimates ranged from -26.66% to 22.39% (Table B5).

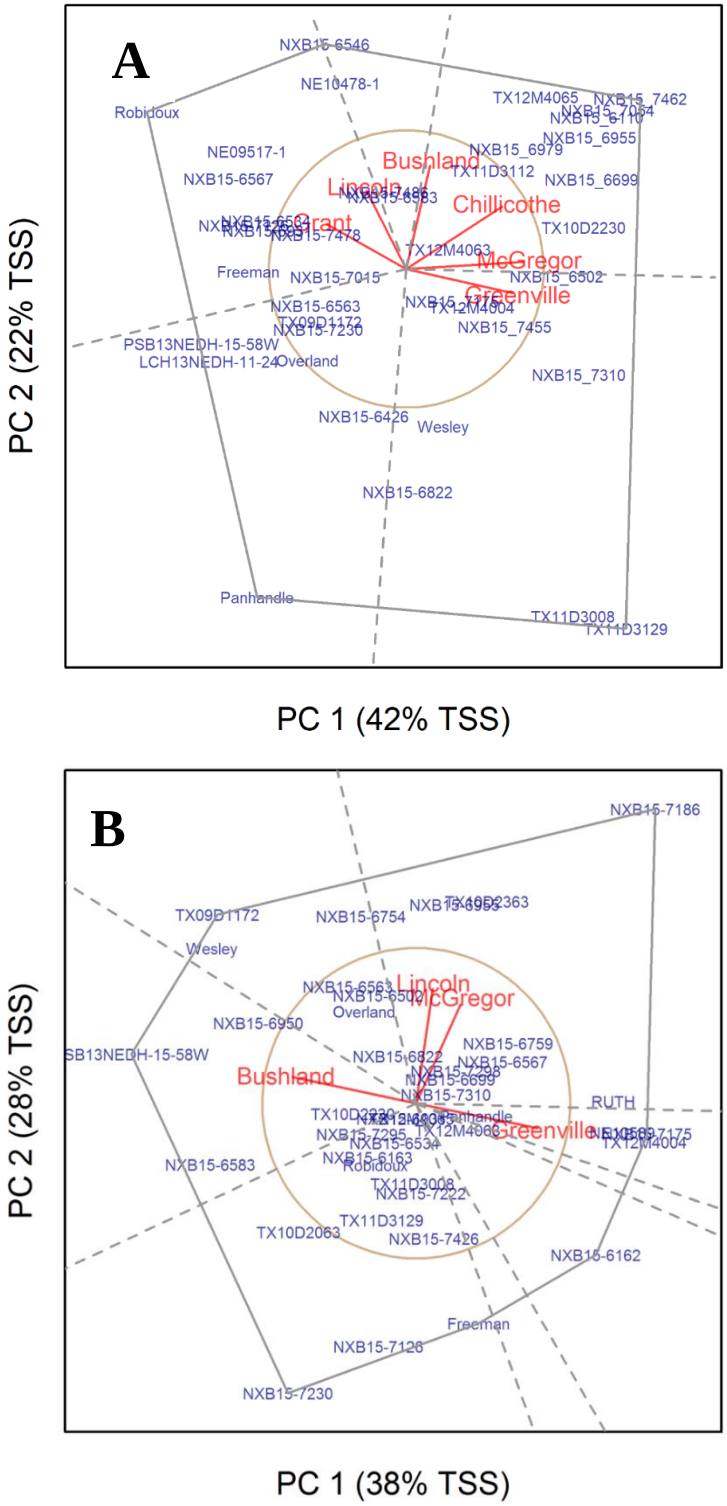

Figure 10 GGE biplot analysis of F2 experiments in 2017 (A) and 2018 (B).

Table 7 Pearson's correlations between combined estimates of F_2 heterosis with combined F_1 heterosis from 2016-2017 experiments in Texas locations.

Experiment	Mid-parent heterosis	High-parent heterosis
2017		
Bushland	0.02	-0.03
Chillicothe	0.41*	0.35*
Greenville	0.14	0.13
McGregor	0.57**	0.58**
Lincoln	0.05	0.04
Grant	-0.14	-0.17
Combined	0.61**	0.58**
TX group	0.68**	0.66**
NE group	0.01	0.01
2018		
Bushland	-0.38	-0.27
Greenville	0.09	0.06
McGregor	0.31*	0.16
Lincoln	0.02	0.01
Combined	0.31*	0.21
Group I (LN, MCG)	0.28	0.19

** $P \leq 0.001$

* $P \leq 0.05$

The heterosis estimates between F_1 and F_2 were negatively to highly correlated in 2017 (Table 7). The correlations between F_1 and F_2 MPH ranged from negative ($r = -0.14$) at Grant to ($r = 0.68$, $P \leq 0.001$) in the TX group. Similar trend was observed in HPH in 2017, where correlation between F_1 and F_2 HPH also ranged from negative ($r = -0.17$) at Grant to ($r = 0.66$, $P \leq 0.001$) in the TX group. High positive correlations were observed between F_1 and F_2 combined (MPH: $r = 0.61$, $P \leq 0.001$; HPH: $r = 0.58$, $P \leq 0.001$) (Table 7). Weak positive correlations were observed between F_1 and F_2 heterosis estimates in 2018. Only MPH estimate from combined analysis of all four locations was significantly correlated with F_1 MPH estimates ($r = 0.31$, $P \leq 0.05$). No significant correlations were observed between F_1 and F_2 HPH estimates in 2018 (Table 7).

At individual crosses level comparisons, crosses heterotic at F2 were found to be heterotic at F_1 stage as well both in 2017 and 2018 experiments (Table 8). Even for crosses where negative heterosis was observed in F_1 stage, the negative heterosis appears to have decreased in F_2 stage (Table 8). Between experiments, the relationship between F_1 and F_2 heterosis was more apparent in 2017 than 2018. Two lines in 2017 (NXB15-6546 and NXB15-7054) and three lines in 2018 (NXB15-6567, NXB15-6754 and NXB15-6563) had negative estimates of heterosis in F_1 but positive estimates in F_2 stage.

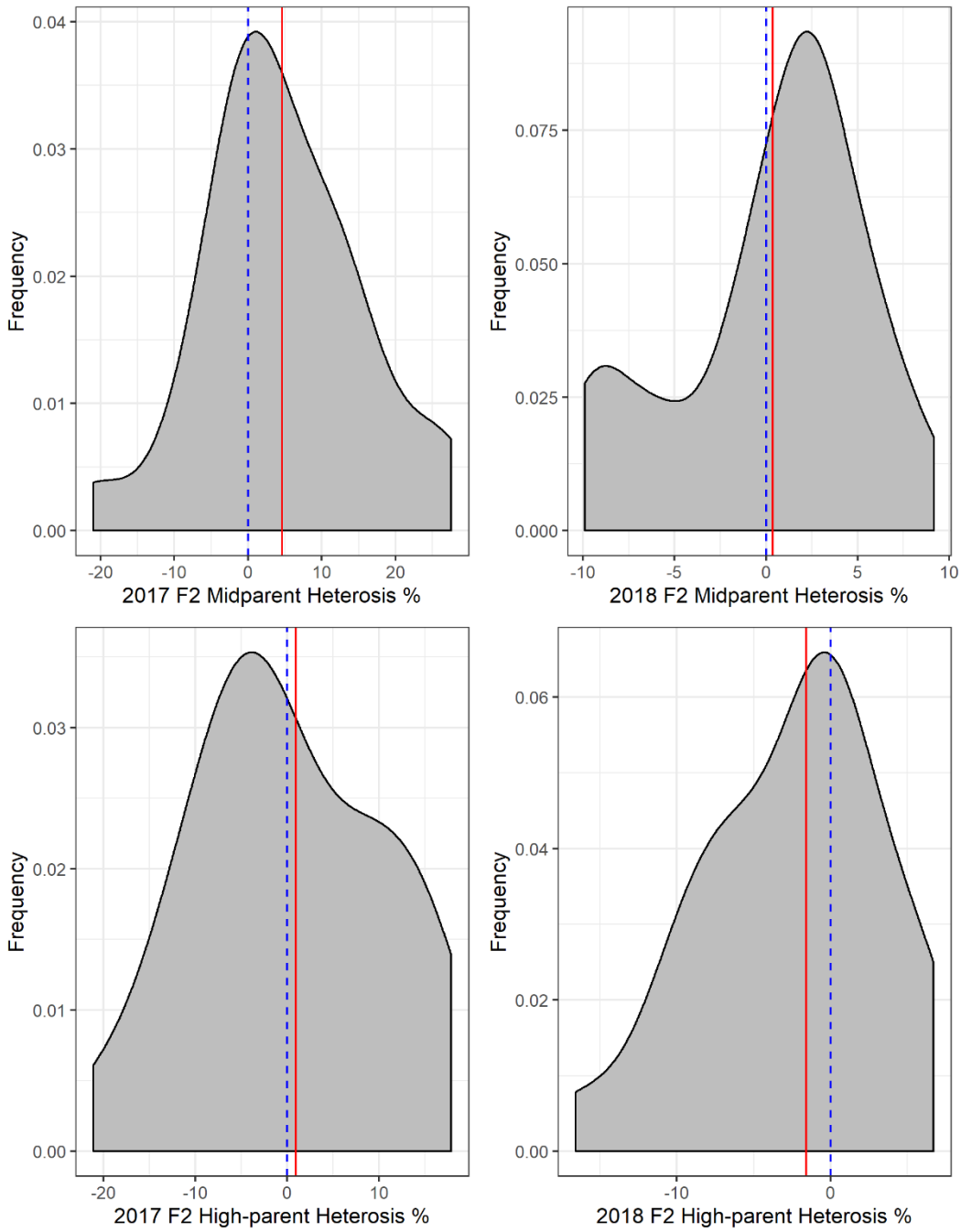

Figure 11 Distribution of mid-parent and high-parent heterosis in the combined analyses of F_2 experiments in 2017 and 2018. The dotted line separates the frequency of lines with positive and negative heterosis whereas the solid line represents average MPH and HPH.

3.4 Discussion

The most important bottleneck in commercialization of hybrid wheat is the lack of cost-effective hybrid seed production system (Mette et al., 2015; Zhao et al., 2015). Limited commercial success in hybrid wheat has been achieved in Western Europe exclusively via use of CHAs for hybrid seed production (Gowda et al., 2012; Longin et al., 2013; Zhao et al., 2015). In a hybrid wheat breeding program, CHAs allow breeders to test any parental combination. Even if cost-effective seed production methods such as cytoplasmic male sterility (CMS) or genetic male sterility (GMS) systems are readily available, breeders will continue to use CHAs for testing hybrid combinations at the early stages of the breeding pipeline. Assuming that CMS or GMS are available, it does not make economic sense, to create parental lines for CMS or GMS and then test them for heterosis and quantitative genetic parameters. The most logical steps would be to test hybrid combinations first, select the best cross combinations and then only advance to converting these parental lines for CMS or GMS systems. However, considering cost in experimental hybrid seed production via use of CHAs, this method severely limits the ability of the breeders to test hybrids in replicated multi-environment trials. Most of the hybrid wheat testing in recent years has been limited to a few locations and mostly in unreplicated experimental designs (Adhikari et al. 2020.; Basnet et al., 2019; Easterly et al. 2020.; Gowda et al., 2012; Longin et al., 2013). This issue is even more magnified in wheat, as heterotic patterns are yet to be established and breeders need to make decisions about which parents to cross entirely based on combining ability estimates (Technow, 2019).

Table 8 Heterosis estimates of hybrid genotypes from combined analyses of F_2 evaluation experiments in Texas and Nebraska.

2017					*2018*				
Genotype	**MPH[1]**		**HPH[2]**		**Genotype**	**MPH**		**HPH**	
	F_1	**F_2**	**F_1**	**F_2**		**F_1**	**F_2**	**F_1**	**F_2**
NXB15-6979	36.86	11.93	34.89	6.46	NXB15-7310	23.86	2.05	22.39	-0.02
NXB15-7462	26.85	24.83	16.97	15.36	NXB15-6502	21.81	0.57	17.95	-1.23
NXB15-7310	23.86	7.98	22.39	-4.60	NXB15-6699	20.71	2.64	16.37	1.46
NXB15-6502	21.81	0.23	17.95	0.10	NXB15-6955	20.64	6.79	15.86	6.68
NXB15-6699	20.71	14.03	16.37	10.43	NXB15-7186	18.58	5.89	15.93	0.10
NXB15-6955	20.64	8.54	15.86	8.05	NXB15-7222	13.28	-6.17	13.24	-8.92
NXB15-7455	17.30	18.40	9.29	14.66	NXB15-7175	11.44	2.34	5.15	2.09
NXB15-7175	11.44	2.65	5.15	-12.56	NXB15-6822	9.22	-5.18	7.98	-7.19
NXB15-6822	9.22	-3.73	7.98	-5.35	NXB15-7295	7.48	-0.42	4.70	-4.35
NXB15-6110	8.47	27.52	5.06	17.85	NXB15-7126	4.13	-1.80	-4.63	-5.79
NXB15-7126	4.13	0.06	-4.63	-6.31	NXB15-6950	2.80	5.65	-9.84	5.48
NXB15-6931	2.14	4.30	-7.50	0.54	NXB15-6931	2.14	2.30	-7.50	-1.22
NXB15-6583	0.41	5.43	-8.32	2.09	NXB15-7298	1.52	1.83	-3.59	-6.27
NXB15-6534	0.34	5.25	-8.40	-1.46	NXB15-6583	0.41	3.17	-8.32	-1.94
NXB15-7054	-0.34	13.65	-4.07	12.94	NXB15-6759	0.38	4.29	-2.83	3.02
NXB15-6567	-4.00	-10.03	-14.24	-13.16	NXB15-6534	0.34	-1.81	-8.40	-7.19
NXB15-7230	-10.40	-6.72	-14.70	-8.29	NXB15-6163	-3.39	-8.86	-6.29	-11.04
NXB15-6546	-10.43	12.77	-11.23	7.63	NXB15-6567	-4.00	9.17	-14.24	5.90
NXB15-6563	-16.06	-1.13	-26.66	-4.69	NXB15-6754	-5.61	1.62	-13.94	0.16
NXB15-7486	-27.72	-0.76	-40.04	-2.11	NXB15-6162	-6.08	-0.35	-8.86	-2.16
NXB15-7478	-43.36	-2.93	-45.96	-5.52	NXB15-7230	-10.40	-9.90	-14.70	-16.57
NXB15-6426	-62.85	-20.97	-65.06	-21.07	NXB15-7426	-12.91	-9.34	-17.36	-10.43
NXB15-7015	-76.52	-1.31	-93.54	-11.76	NXB15-6563	-16.06	3.65	-26.66	-0.12
Min	-76.52	-20.97	-93.54	-21.07	Min	-16.06	-9.90	-26.66	-16.57
Max	36.86	27.52	34.89	17.85	Max	23.86	9.17	22.39	6.68
Average	-2.07	4.78	-8.37	-0.03	Average	4.36	0.35	-1.20	-2.59

[1] Mid-parent heterosis

[2] High parent heterosis

In several autogamous crops with perfect flowering systems F_2 have been utilized for estimating heterosis (Ghaderi et al., 1984; Meredith, 1990; Shehata and Comstock, 1971) and quantitative genetic parameters via classical mating designs such as Designs I and II (Comstock and Robinson 1948, 1952). Technow (2019), evaluated the possibility of using F_2 bulk's performance as a proxy for F_1 performance in a genomic prediction scenario. Using simulation models, he concluded that genomic prediction models that are trained on F_2 performance data can produce higher accuracy than models trained on F_1 performance data, depending on heritability estimates. Moreover, genomic prediction accuracies via use of F_2 are going to be higher in crops like wheat, where heterotic patterns are not well defined. Recently, Friedrichs et al., (2016) demonstrated that F_2 yield heterosis can be used to select parental cross combinations with high genetic variances in a soybean line breeding program. Individual line selection from crosses with high mid-parent F_2 heterosis can lead to future higher yielding varieties in pureline soybean breeding (Friedrichs et al., 2016). Similar results were also reported in durum wheat (*Triticum durum* L.) by Kotzamanidis et al. (2008), where F_2 mid-parent heterosis was more effective than molecular markers at predicting promising crosses. Same strategy of using F_2 heterosis for predicting better performing parental cross combinations has been utilized in barley (*Hordeum vulgare* L.) (Immer, 1941; Kotzamanidis and Roupakias, 2004) and durum wheat (Knott, 1994; Kotzamanidis et al., 2008). In a hybrid crop breeding scenario, F_2 performance has also been effectively utilized for heterosis and combining ability estimation in flax (Shehata and Comstock, 1971), cotton (Meredith Jr and Brown, 1998; Meredith, 1990; Tang et al., 1993; Wu et al., 2004), maize (Flachenecker et al., 2006; Troyer, 1986), triticale (Oettler et al., 2001), rapeseed (Lefort-Buson et al., 1986), dry beans (Ghaderi et al., 1984), peanuts (Isleib and Wynne, 1983) etc. Therefore, using F_2 performance as a proxy for F_1 performance, combining

ability or selecting parents for crossing is a proven concept, performed in a variety of crop species. Due to the challenges currently faced by hybrid wheat breeding programs such as low amount of seed produced per plant, expensive seed production and lack of well-defined heterotic groups utilizing F2 data for getting estimates of F1 performance is a very promising strategy.

Several previous studies have estimated heterosis and combining ability using F2 data in bread wheat (Cox and Murphy, 1990; Cregan and Busch, 1977; Harrington, 1940; Joshi et al., 2004). However, most of them have few parents and hybrids evaluated and are mostly space planted which skews the heterosis estimates. In 116 F_2 populations resulting from crosses between hard red winter (HRW) and soft red winter (SRW) wheat cultivars from US and international cultivars, (Cox and Murphy, 1990) estimated an average F_2 grain yield MPH of -2.4 – 8.6%. The highest average MPH was observed in crosses between cultivars outside of US. The most relevant comparison between the (Cox and Murphy, 1990) study and this study can be made with their heterosis estimates from HRW x HRW crosses which included lines from UNL and TAMU. The average MPH in the HRW x HRW crosses was 2.3% which is comparable to this study; 4.35% (2017) and 0.35% (2018). Winzeler et al. (1993) also reported positive F_2 MPH in crosses between bread wheat and spelt wheat.

In this study, we report F_2 MPH as high as 27.52% in 2017 and 9.17% in 2018. Similarly, HPH estimates were as high as 17.85% in 2017 and 6.68% in 2018. However, our main objective was to compare the relationship between F_1 and F_2 heterosis and make inferences about the possibility of using F_2 heterosis for F_1 performance. The F_2 heterosis was found to be low to moderately correlated with F_1 heterosis and more so for MPH than HPH (Table 7). No significant correlations were observed between HPH in 2018. G × E appeared to have an impact on the correlations between F_1 and F_2 heterosis since, the correlations were higher in TX group

as compared to all locations combined and NE group. In both experiments, F_1 with positive MPH estimates had positive MPH in F_2 stages as well. The correlation between F_1 and F_2 HPH was bit lower than MPH in both experiments (Table 7). However, yield and heterosis estimates between single locations were not very promising. This suggests that F_2 testing should be done in multiple locations to make meaningful comparisons. This is most likely due to F_2 hybrids being heterogeneous (Meredith, 1990).

The results from this study provide evidence that F2 testing can be used to supplement F1 testing in hybrid wheat breeding programs. Breeders need to decide how and when F_2 testing can be integrated in hybrid wheat breeding. The most logical stage to integrate F_2 testing in hybrid wheat breeding program would be as right after preliminary testing of F_1 hybrids (Figure 12). F_2 evaluation can also be extended to multiple years at this stage to select for yield stability across years. Data from F_2 testing can be used to further select and reduce the number of F_1 to be evaluated at advanced yield trials (AYTs). Relatively few hybrid cross combinations selected from F_2 evaluations would be advanced to AYTs. These AYTs would be METs spanning multiple years and replicated trials. A larger crossing block with these limited cross combinations needs to be planted after PYTs and before AYTs to produce enough hybrid seed for METs. Moreover, genomic predictions could be used using F2 or F1 data to select the best cross combinations for the advanced crossing blocks. Seed production at this stage could use any seed production methods such as CHAs, CMS or GMS, if they are available. A subsidiary line conversion program would be appropriate that would start from this stage.

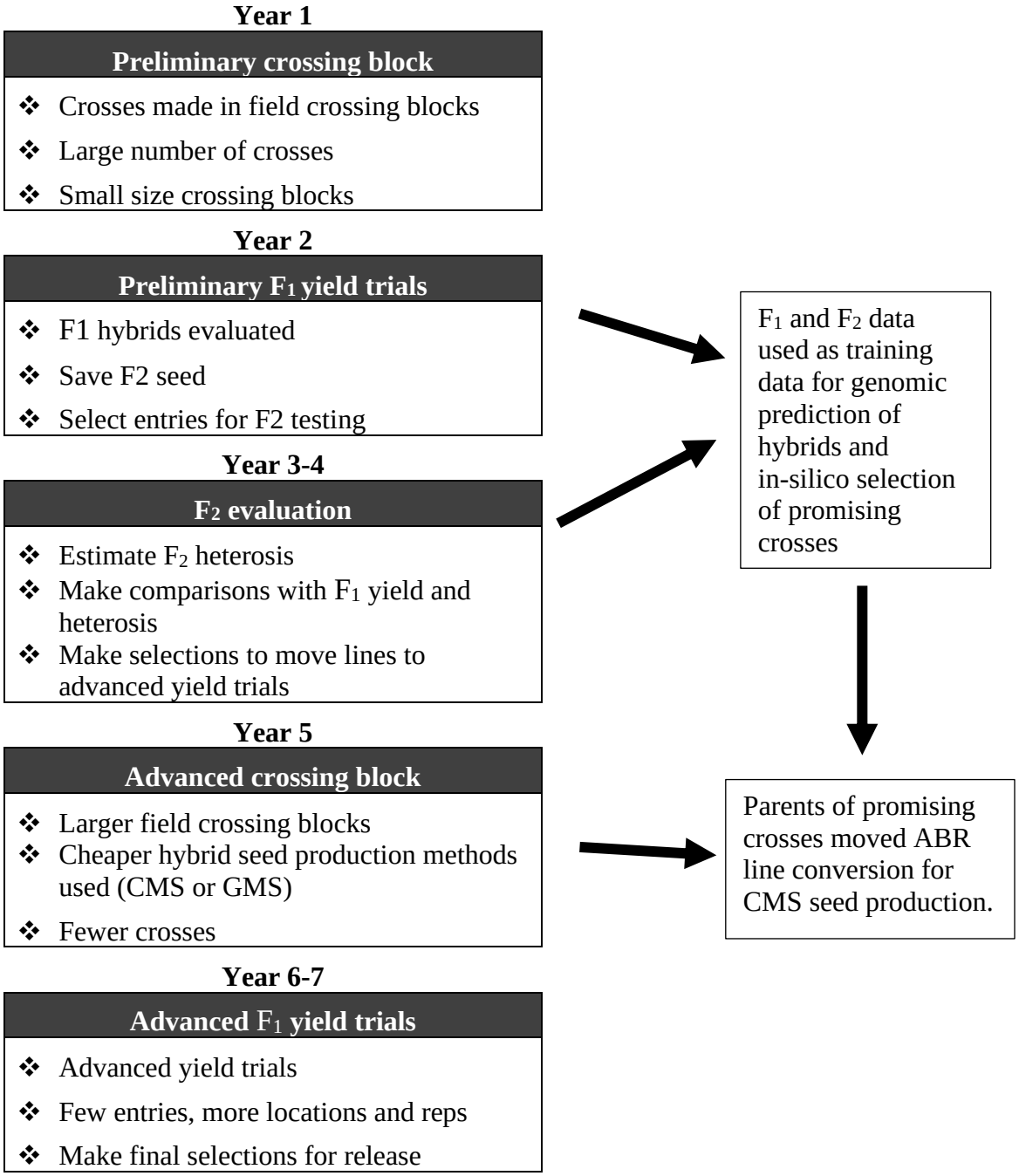

Figure 12 Schematic diagram depicting integration of F_2 evaluation in a hybrid wheat breeding pipeline.

3.4 Conclusions

Hybrid wheat seed production at the early stages of the pipeline is very expensive, due to the high number of crosses that need to be made. Due to the ease of turning any high yielding line into female parent, CHA is most likely going to be the method of choice for hybrid seed production at early stages, which makes seed production even more expensive. This limits the breeding programs ability to test hybrids in METs and make informed selection decisions. F_2 testing can be effectively utilized to support decision making in selection of F_1 hybrids for advanced yield trials. Based on the data presented in this study, hybrid wheat candidates with positive MPH and HPH at F_2 stage are very safe candidates for further evaluations in a hybrid wheat breeding program.

CHAPTER IV

GENETIC CONTROL OF FERTILITY RESTORATION IN A CYTOPLASMIC MALE STERILITY SYSTEM

4.1 Introduction

Inexpensive hybrid seed production is the most important aspect of hybrid wheat (*Triticum aestivum* L.) breeding which can be done by use of cytoplasmic male sterility (CMS) and its corresponding nuclear restorer source (*Rf*), popularly known as the three-line system of hybrid seed production. CMS is available in several wheat relatives, and it can readily be backcrossed into suitable lines for hybrid breeding and seed production. However, finding sources that restore full fertility on consistent basis has always been a challenge and it is the single most limiting factor in use of the three-line seed production system in hybrid wheat breeding (Virmani and Edwards, 1983). Interests on using CMS wheat for hybrid seed production started with the reporting of CMS in *Triticum aestivum* by Japanese scientists in 1951 using cytoplasm of *Aegilops caudata* L (Kihara, 1951) and *Triticum ovata* in 1955 (Fukasawa, 1953). Later, CMS wheat was developed by using cytoplasm of *Triticum timopheevi* by Wilson and Ross in Nebraska in 1962 which was found to be most suitable for hybrid seed production (Wilson, 1962; Wilson and Driscoll, 1983). Fertility restoring lines for CMS wheat lines with *T. timopheevi* was first reported by Schmidt in 1962 (Schmidt, 1962) and later by Livers in 1964 (Livers, 1964). Livers reported that fertility restoration was possible by transferring *Rf* genes from *T. timopheevi* to common wheat and using it as a male parent (Livers, 1964). He reported that fertility restoration was conditioned by two dominant restorer genes *Rf1* and *Rf2*, but their chromosomal location was unknown (Livers, 1964). This initiated great interest in genetic mapping of these *Rf* genes.

Robertson and Curtis (1967) conducted the pioneering work of mapping *Rf* genes using monosomic lines. They mapped the *Rf* gene *Rf1* in chromosome 1A in a line developed by Livers (1964) and reported other modifier genes in 1B, 2A, 3D, 6A and 6B. Similar study was done by Yen et al. (1969) in which they mapped two major genes, *Rf1, Rf2*, and two minor genes in 6B and 6D. *Rf* genes were later reported on 1A, 1B, 4D, 5D, 6B, 7B and 7D on six sources of fertility restorer genes via monosomic analysis (Bahl and Maan, 1973; Du et al., 1991; Maan, 1985). Of these genes, *Rf1* in 1A, *Rf3* in 1B and *Rf4* in 6B were considered to be major genes whereas all other were considered to be modifier genes that interact with the major genes in restorer line R113 (Bahl and Maan, 1973; Du et al., 1991; Maan, 1985). Mapping efforts of fertility restoration were carried out using SSR molecular markers in the 1990s. *Rf3* was mapped in line R113 (Ma and Sorrells, 1995) and in 'Chinese spring' (Ahmed et al., 2001; Kojima et al., 1997) and *Rf4* was mapped in line R113 (Ahmed et al., 2001; Ma and Sorrells, 1995) using Restriction Fragment Length Polyporphism RFLP markers. Similarly, *Rf3* was also mapped to chromosome 1B using SSR markers (Zhou et al., 2005) and in addition to the known *Rf* genes, a new fertility restorer gene in chromosome 1DS was mapped in line PWR4099 using SSR markers which was named as *Rf8* (Sinha et al., 2013). A few recent studies have tried to map restorer genes in previous sources (*Rf1, Rf3* and *Rf4*) of fertility restoration using modern mapping methods and next generation sequencing markers (Geyer et al., 2018; Geyer et al., 2016; Würschum et al., 2017). Despite the wealth of these molecular mapping studies, there has not been a consensus on how many genes are needed to restore full fertility in CMS wheat (Bahl and Maan, 1973; Geyer et al., 2016; Miller et al., 1974). Previously identified restorer sources have been known to restore fertility only partially and result in partially sterile hybrids (Hayward, 1975; Keydel et al., 1979; Virmani and Edwards, 1983), which has limited their use

in hybrid wheat breeding. This was one of the major reasons why CMS system has been of limited use in hybrid wheat breeding (Virmani and Edwards, 1983; Whitford et al., 2013). Lack of breeder friendly markers tightly linked with *Rf* genes have limited the efforts to stack these *Rf* genes in a single line to make them suitable for hybrid seed production in CMS system (Geyer et al., 2016).

This study was aimed at identifying the genes that provide full fertility restoration in an Australian source 'Cargill115', tag those genomic regions with Single Nucleotide Polymorphism (SNP) markers and identify candidate genes. Development of functional KASP markers arising from the SNP tags, can facilitate the stacking of *Rf* together to develop male lines that restore full fertility in the three-line system of hybrid seed production; while the identification of candidate genes can aid in fine mapping and cloning of these genes.

4.2 Materials and methods

4.2.1 Mapping population development

The restorer line (Cargill115) was used as the female since it has a CMS cytoplasm and crossed with a CIMMYT spring wheat line C80 as male. F_1 progeny was selfed and the F_2 population was advanced to $F_{4:5}$ to produce a population of 299 recombinant inbred lines (RILs). Using Cargill115 as female allowed the transfer of CMS cytoplasm to the RILs and facilitated evaluation of restorer genes in presence of CMS cytoplasm. The RILs *per se* were phenotyped (called RILs phenotyping hereafter) for their capacity to form fully fertile spikes. In addition, in 2018 the RILs were also crossed with a CMS tester line (A line of the ABR system) to produce 299 $F_{4:5}$ test cross hybrids (called RILs test cross phenotyping hereafter) (Figure 13). Phenotypic evaluation for fertility restoration was conducted on both RIL population and RIL test cross population.

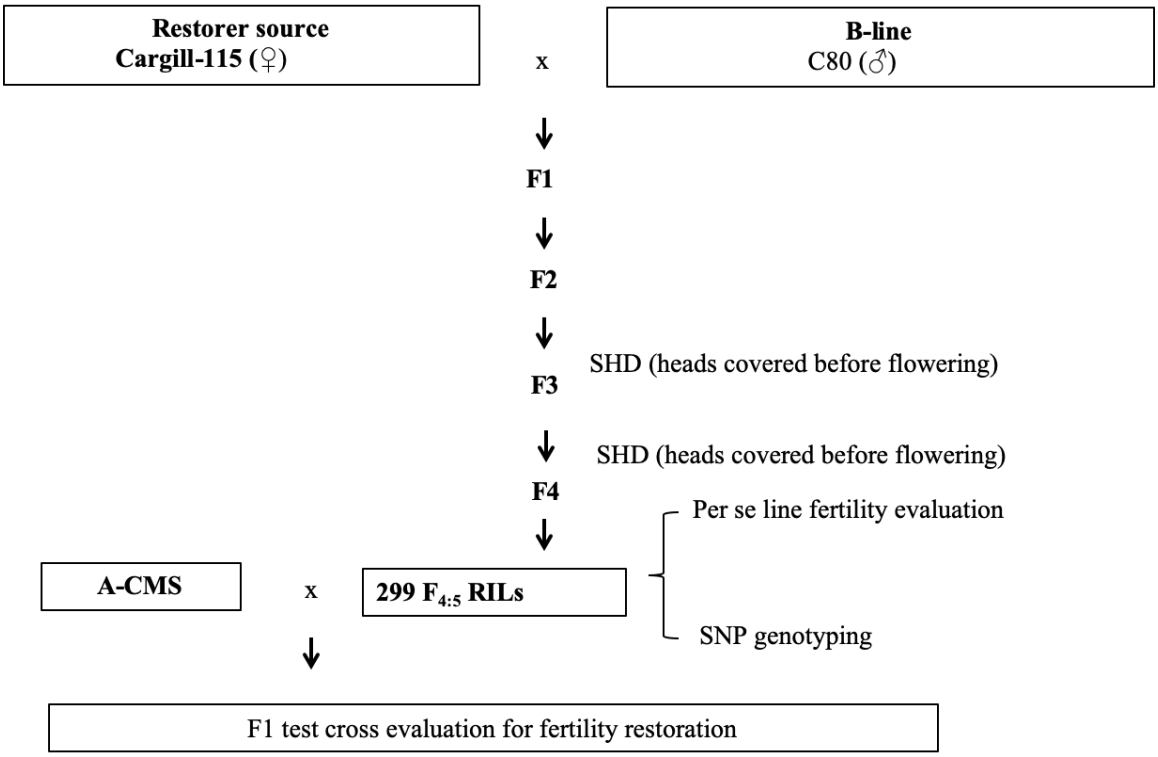

Figure 13 Schematic diagram of the recombinant inbred line (RIL) population development. Per se line fertility evaluation refers to fertility evaluation of RILs themselves.

4.2.2 Phenotyping for fertility restoration

The RILs population was planted in a greenhouse inside CIMMYT campus in El Batan, Mexico in 2018. After heading and before anthesis, five heads from each line were covered with a paper bag to prevent pollen contamination and ensure selfing. Upon maturity, a visual 1-10 score was given to the RILs where 1 was completely fertile and 10 was completely sterile. After the visual scoring, the five heads were individually harvested and data was collected on number of spikelets per head and total seed set per head.

In 2018, the RILs test cross progenies were planted in field in El Batan, Mexico and evaluated for fertility restoration. Upon maturity, a visual score of 1-10 was given to the test cross progenies. In addition, ten spikes chosen at random were harvested upon maturity, to collect data on number of sterile spikelets per head, sterility percentage and total seed set. An average value of ten spikes was used to conduct linkage mapping.

In 2019, the test cross progenies were planted in replicated trials in three locations in Mexico in Obregon, Toluca and El Batan. The experiments planted in 2019 were laid out in a randomized complete block design with two replications. Visual score for sterility was calculated from all test cross evaluations. In addition, seed per spike data was collected from five spikes in Obregon.

Broad sense heritability was calculated using the formulae $h^2 = \sigma^2_G / (\sigma^2_G + \sigma^2_e/r)$, where σ^2_G is the genetic variance associated with RILs and σ^2_e is the error variance and r is the number of replications. Heritability calculations were done using variance components estimated using PROC MIXED procedure in SAS 9.4.

For combined analysis of visual score and seed per spike, best linear unbiased estimates (BLUEs) were calculated in SAS 9.4 using PROC GLM procedure. For combined analysis of visual score, test cross visual score data from El Batan (2018, 2019), Toluca and Obregon were used whereas for seed per spike data, data from Obregon and El Batan (2018) was used.

4.2.3 Genotyping and genetic linkage map construction

Plant leaf tissue was harvested from the RILs planted in CIMMYT greenhouse in 2017 at maturity and sent to TraitGenetics (Gatersleben, Germany) for a 15K + 5K Infinitum® iSelect® array containing 17,267 single nucleotide polymorphism (SNP) markers combined from the 90K iSelect array (Wang et al., 2014) and the 820K Axiom® array (Winfield et al., 2016). The

markers in the genotypic data were screened for monomorphism and minor allele frequency (<5%). The chromosome information of the markers was extracted from wheat reference genome (RefSeq v1.0) from the International Wheat Genome Sequencing Consortium. The SNP markers were grouped according to their respective chromosome information and used for linkage map construction via 'onemap' package (Margarido et al., 2007) in R version 3.5. Markers within each chromosome were linked and ordered using an independence LOD threshold of 10.0 and genetic distances between them was calculated using Kosambi function.

4.2.4 Linkage mapping and QTL analysis

Linkage mapping was conducted in R package 'qtl' for each individual trait using a composite interval mapping algorithm using significant markers as cofactors (Broman et al., 2003). Significance thresholds of LOD scores for each trait were identified via 10,000 permutation tests.

The markers closest to the QTL peak and their Bayesian confidence interval were calculated using "bayesint" function in "qtl" package (Broman et al., 2003).

4.2.5 KASP™ genotyping

Tightly linked SNPs with significant QTLs were converted to the KASP platform. SNPs that were consistently identified as significant in different traits were used to design KASP markers using Primer3 software (http://biotools.umassmed.edu/bioapps/primer3_www.cgi). Each KASP reaction was performed in a volume of 10 mL with 5 mL of DNA and 5 mL of the prepared genotyping mix (2´ KASP master mix and primer mix) following the protocols for the preparation and running of KASP reactions from the KASP manual (http://www.kbioscience.co.uk). Amplification was performed using the ABI 7500 instrument

(Applied Biosystems) following protocols described in these studies (Tan et al., 2017a; Tan et al., 2017b).

4.2.6 Candidate genes identification

For candidate gene identification, physical positions of flanking SNPs within the QTL interval were from the wheat reference genome (Refseq v1.0). All the predicted genes and their physical positions within the segment identified by the flanking SNPs were extracted from the reference genome using the high confidence annotation (HighConfidenceGenesv1.1).

Pentatricopeptide repeats (PPR) gene family has been identified as the family of genes responsible for fertility restoration in other hybrid crops. A track called "Pentatricopeptide repeats (PPR) gene family" was selected in JBrowse to identify manually curated genes of PPR gene family from the region flanked by the two SNPs. These genes are considered to be the high confidence candidate genes for fertility restoration. So, nucleotide sequence of these genes was used to search the NCBI database to identify orthologues and homologues in other species to infer their function.

4.3 Results

4.3.1 Trait evaluation of the RIL population

In El Batan RIL per se phenotyping, the average seeds per spike calculated from five bagged spikes, ranged from 5 to 65 with a mean of 35.6 seeds while the number of sterile florets ranged from 0 to 30 with a mean of 9.6 (Figure 14). The visual score for RILs in El Batan ranged from 1 to 8 with an average score of 2.58, where 1 is fully fertile and 10 is fully sterile.

4.3.2 Test cross evaluation of RILs

In the test cross evaluation in El Batan in 2018, the average seeds per spike calculated from ten bagged spikes per genotype before flowering ranged from 0 to 70 with a mean value of

24.31 (Figure 14). The average number of sterile spikelets per spike calculated from ten bagged spikes in test cross evaluation ranged from 0 to 45 with a mean value of 19.79. The visual score for fertility restoration in test cross evaluation ranged from 1 to 10 with an average score being 5.45. About 50 RILs had zero sterile florets in test cross evaluation in El Batan, which indicates complete fertility restoration (Figure C4).

In test cross evaluation in Obregon in 2019, seed per spike ranged from 0 to 90 with a mean of 49.45 seeds per head. The visual score ranged from 1 to 10 with a mean value of 3.34. In test cross evaluations in Toluca 2019, visual scores ranged from 1 to10 with a mean of 5.78 while in El Batan 2019, the visual scores ranged from 1 to10 with a mean of 5.38 (Figure 14, Table 9).

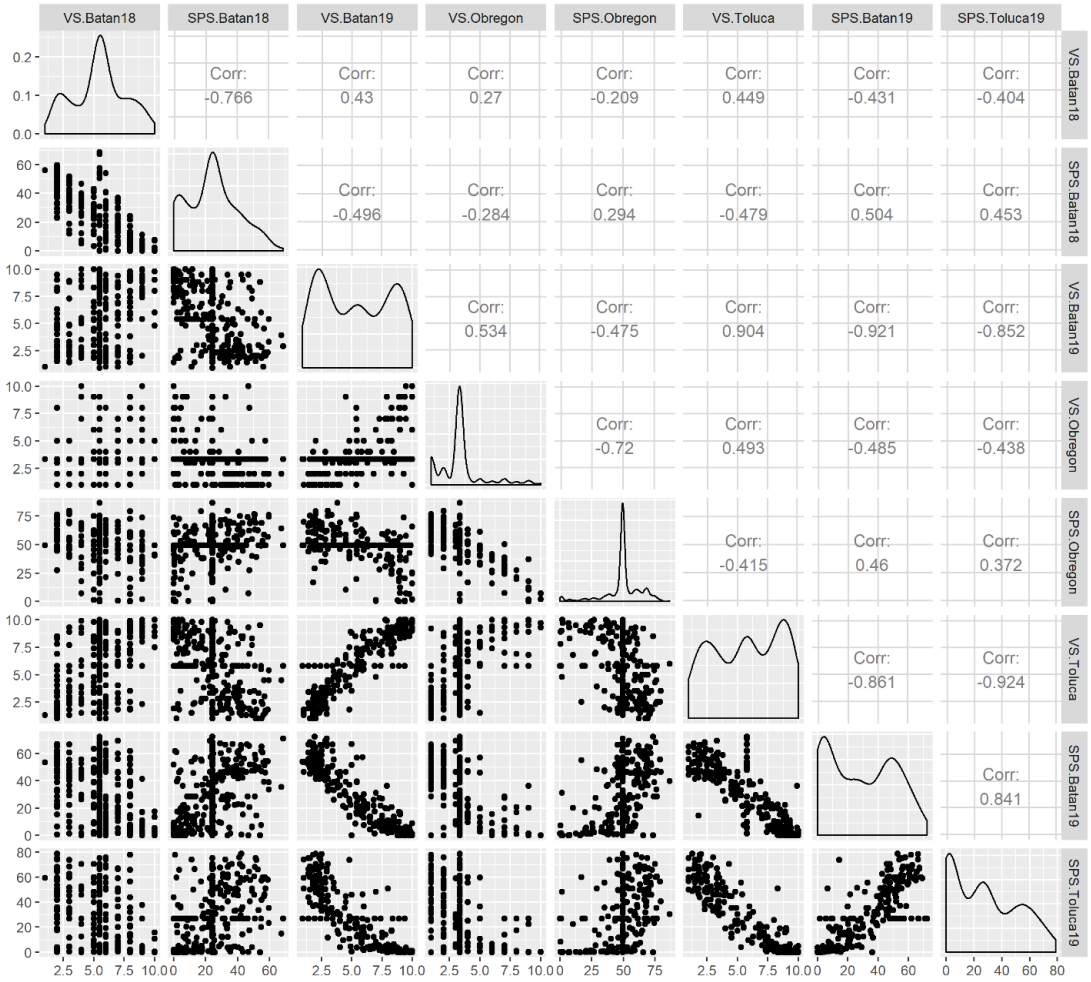

Figure 14 Phenotypic distribution of traits and correlation between the traits. The traits are Visual sterility score El Batan, Mexico 2018, Visual sterility score El Batan 2019, Visual sterility score Obregon, Mexico, Seed per spike Obregon, Sterile florets El Batan 2018 and seed per spike El Batan 2018, respectively along the diagonal.

4.3.3 Correlations between traits and heritability

Statistically significant correlations ($P \leq 0.001$) were observed between visual scores in the five environments (r = 0.56 to 0.95) (Fig. 14). Based on these high correlations, a combined analysis of all five environments considering genotypes as fixed and environments as random was conducted in SAS 9.4 using PROC GLM. The least square means were named combined visual score and it was also used as a trait for mapping. The traits combined across locations were seeds per spike and visual score. Similarly, statistically significant correlation (r = 0.46, $P \leq 0.001$) was observed between seed per spike in Obregon and El Batan test cross evaluation in 2018. A combined analysis of these two traits were also conducted in SAS 9.4 (SAS Institute, 2017) to calculate combined seed per spike which was also used for mapping. Heritability calculated on an entry mean basis ranged from 0.74 to 0.76 in RILs evaluation and 0.94 to 0.97 in test cross evaluations (Table C2). In the combined analysis of test cross evaluation, heritability for visual score was 0.93 and 0.61 for seed per spike.

4.3.4 Marker data and linkage groups

A total of 6264 markers were polymorphic with a minor allele frequency of > 5%. After additional filtering based on segregation distortion, 3047 markers were assigned to 37 linkage groups (Table C3). The length of individual linkage groups ranged from 12.96 cM to 206.80 cM and number of markers within an individual linkage group ranged from 21 to 268. The total length of the linkage map was 2990.28 cM with an average marker density of 0.98 marker per cM of genetic distance.

4.3.5 Linkage mapping and QTL analysis

One minor effect QTL was identified in chromosome 5A (R^2 = 6.4 to11.0%) in RILs phenotyping in El Batan 2018 (Figure 15, Table 9). The source of the QTL is the non-restorer parent C80. The same QTL was also detected in test cross phenotyping in El Batan 2018.

A major effect QTL was identified in chromosome 1B in all test cross evaluations (Figure 16, Table 9). The QTL was consistently identified at the same genomic regions across experiments and traits. The source of the QTL is the restorer parent Cargill 115 and it explains about 35 to 45 % phenotypic variance. In addition to this major QTL, a minor effect QTL was identified in chromosome 6B, consistently across traits and experiments. The minor effect QTL explains about 3.4 to 7.1% of the phenotypic variance and appears to be originating from non-restoring parent C80. Markers closest to the LOD peak and the associated marker effects in fertility restoration are also reported in Table 9.

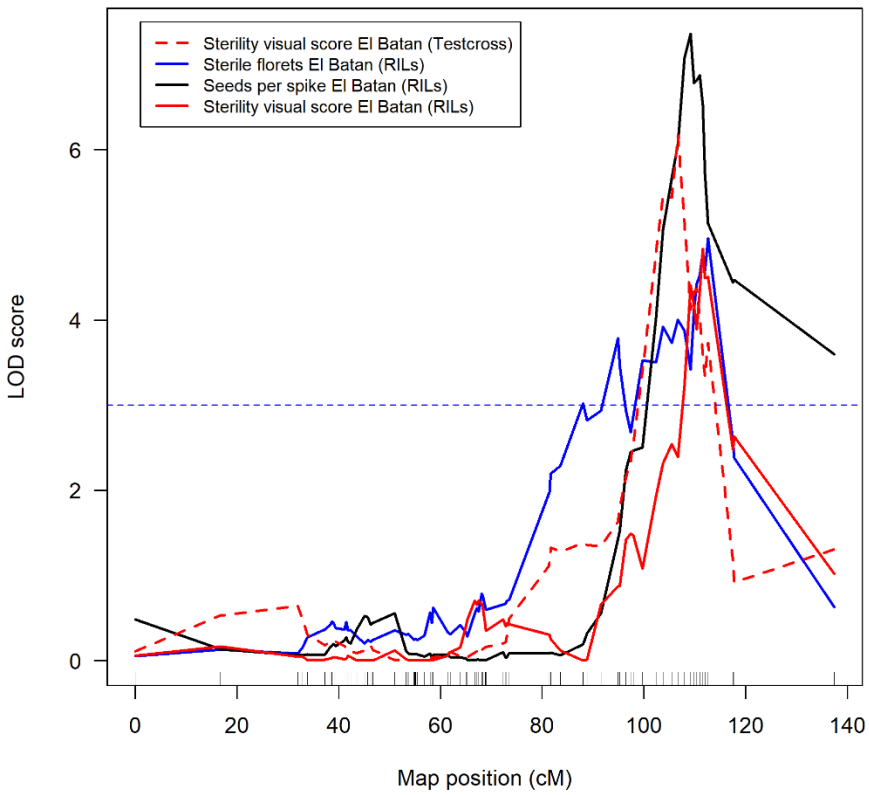

Figure 15 QTL identified on chromosome 5A in recombinant inbred line evaluation in El Batan, Mexico in 2018

Table 9 Quantitative trait loci identified with their corresponding flanking marker information from the three phenotyping experiments conducted for evaluation of fertility restoration in a recombinant inbred line population developed from a restorer source 'Cargill 115' at the International Maize and Wheat Improvement Center (CIMMYT), Mexico.

Traits	Environment	CHR	LOD	R^2	QTL effects	Peak (cM)	Physical position (Mb)	Closest to the peak	QTL Interval
Visual score	Toluca	1B	35.2	45.0%	-2.2	157.6	18.41	Tdurum_contig78972_316	157.6-157.6
Visual score	Obregon	1B	11.7	30.0%	-1.6	162.2	16.85	AX-94769850	157.6-162.2
Visual score	El Batan 2019	1B	41.0	46.0%	-2.2	157.6	18.42	AX-94569385	157.6-158.6
Visual score	El Batan 2018	1B	17.5	34.0%	-1.7	157.6	18.41	Tdurum_contig78972_316	157.6-157.6
Visual score	Combined	1B	42.9	47.0%	-2.0	157.6	18.41	Tdurum_contig78972_316	157.6-157.6
Seed per spike (test cross)	Obregon	1B	8.5	21.0%	9.7	158.6	19.04	D_F5XZDLF02I14SZ_56	157.6-162.2
Seed per spike (test cross)	El Batan 2018	1B	41.0	36.0%	13.0	157.6	18.41	Tdurum_contig78972_316	157.6-157.6
Seed per spike (test cross)	Combined	1B	23.9	35.0%	12.0	157.6	18.41	Tdurum_contig78972_316	157.6-158.6
Visual score	El Batan 2019	6B	7.1	9.2%	1.1	4.6	20.71	AX-94537931	4.6-4.9
Visual score	Toluca	6B	5.2	8.6%	1.0	4.9	4.87	AX-94757558	2.1-4.9
Visual score	Obregon	6B	3.5	8.7%	0.8	4.6	20.71	AX-94537931	0.4-4.7
Visual score	Combined	6B	6.8	7.5%	0.8	4.6	20.71	AX-94537931	4.6-4.9
Seed per spike (test cross)	Obregon	6B	3.4	8.6%	-6.3	4.9	4.87	AX-94757558	0.0-4.9
Seed per spike (test cross)	El Batan	6B	6.1	7.2%	-5.4	4.6	20.71	AX-94537931	4.6-4.9
Seed per spike (test cross)	Combined	6B	5.2	9.2%	-5.8	4.9	4.87	AX-94757558	0.0-4.9
Seed per spike (RILs)	El Batan	5A	7.1	11.0%	-3.9	106.7	-	TG0020	105.5-110.9
Sterile florets (RILs)	El Batan	5A	4.6	6.4%	-1.3	112.6	598.66	IAAV4799	91.6-112.6
Visual score (RILs)	El Batan	5A	5.0	7.3%	0.5	111.6	596.01	BS00100510_51	109.21-112.61
Visual score (test cross)	El Batan 2018	5A	6.7	11.0%	0.9	106.7	-	Excalibur_rep_c111129_125	102.4-107.9

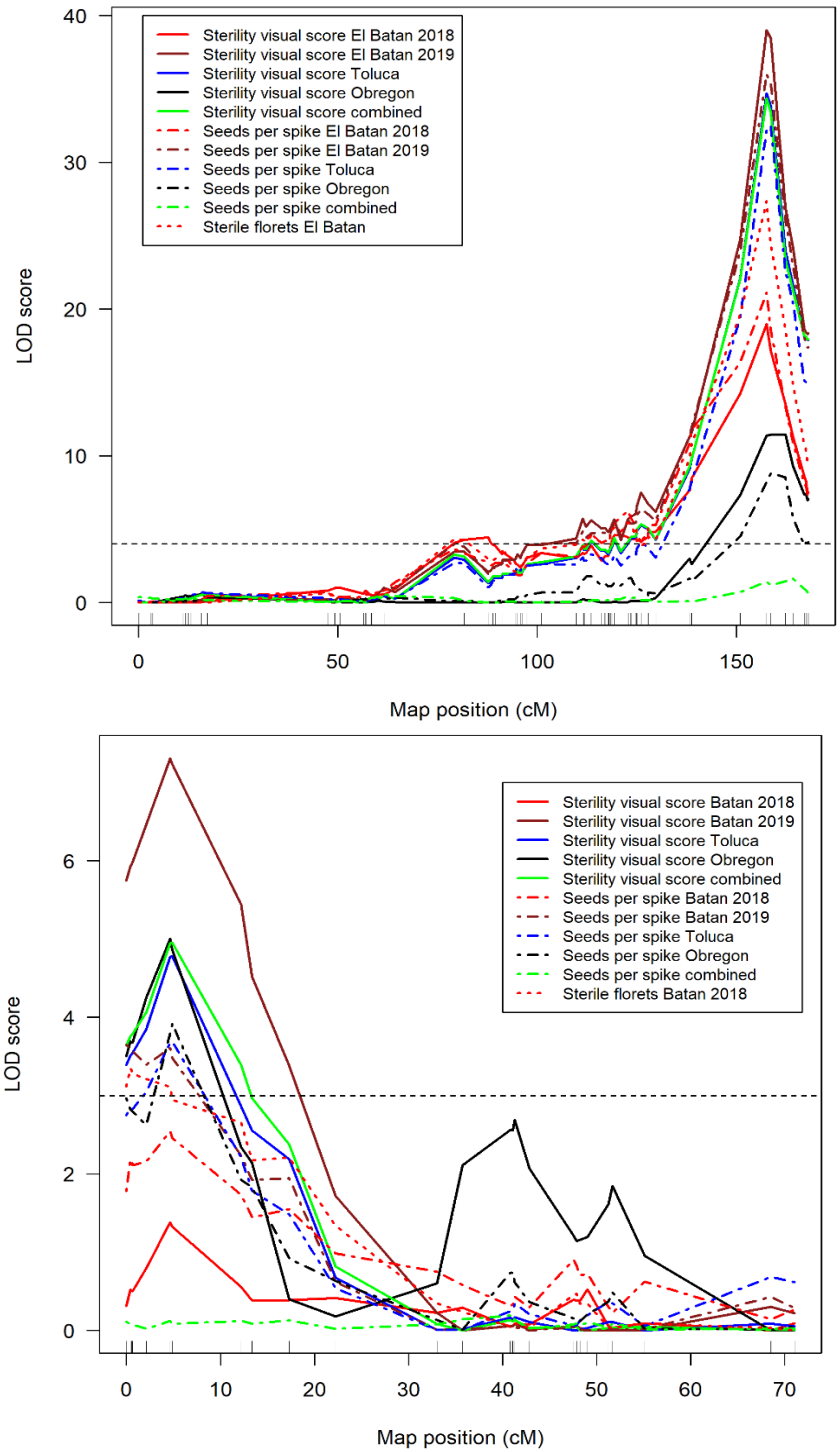

Figure 16 QTL plots in chromosome 1B (upper panel) and 6B (lower panel) with LOD score in y-axis and map position of markers in x-axis. The color and line type of LOD curve represent traits (seeds per spike, visual sterility score and sterile florets) used for QTL mapping. The phenotypic data came from phenotyping experiments in El Batan, Toluca and Obregon from 2017 to 2019.

A stepwise QTL model was implemented in R using function “fitqtl” to test for interaction effects of minor QTLs 5A and 6B with major effect QTL in 1B. Results of this stepwise QTL model indicated a positive significant interaction of QTLs in 6B and 1B but the interaction between 5A and 1B were not significant (Table 10). The QTL model with QTL in 1B and 6B combined explained about 41 to 53 % phenotypic variances in combined analysis for visual score and seed per spike whereas the QTL model with 1B and 5A combined explained about 40% of the genetic variance.

4.3.6 KASP genotyping using flanking markers of major QTLs

KASP primers were designed for the major QTL in chromosome 1B using one SNP tagging the QTL and two SNPs flanking the QTL (Table 11). All three KASP assays were successful in differentiating the RILs into the respective genotype groups. The KASP markers were also tested on an unrelated population of 15 spring and winter wheat lines from CIMMYT and Texas A&M line breeding. The unrelated population included another restorer line (Cargill 116), capable of full fertility restoration in CMS lines based on field evaluations (Bhoja Raj Basnet, personal communication). The KASP markers were successful in identifying the lines with the presence and absence of this QTL.

Table 10 Additive interaction effects of QTLs identified in chromosome 1B, 6B and 5A estimated by analysis of variance. The combined visual score and seed per spike data used for QTL analysis came from phenotyping experiments in El Batan (2018 and 2019), Toluca (2019) and Obregon (2019). The sterility visual score for recombinant inbred lines (RILs) came from phenotyping for RILs in El Batan 2018.

Trait	**QTL Model**	**R^2**	**QTL effects**	**F test Significance**
Combined visual score	1B + 6B + 1B × 6B	53.4		
	1B	45.9	-1.81	***
	6B	8.4	0.53	***
	1B × 6B	2.8	0.39	***
Combined seed per spike	1B + 6B + 1B × 6B	40.8		
	1B	31.8	9.8	***
	6B	7.0	-4.0	***
	1B × 6B	1.4	-2.2	*
Sterility visual score (RILs)	1B + 5A + 1B × 5A	39.9		
	1B	28.9	-1.35	***
	5A	7.9	0.7	***
	1B × 5A	0.9	0.2	

*** F test significant at $P \leq 0.0001$ level

* F test significant at $P \leq 0.01$ level.

Table 11 Kompetetive Allele Specific Primers (KASP) designed for single nucleotide polymorphism markers tightly linked with a major QTL identified in chromosome 1B in Cargill 115.

Marker name	Primer ID	Primer sequence
BS00089524_51	Common reverse	ATTACAGTGCGCTTGTCG
	Allele 1	GAAGGTGACCAAGTTCATGCTTTGACAGTCAGAAAGGACCAT
	Allele 2	GAAGGTCGGAGTCAACGGATTTGACAGTCAGAAAGGACCAG
Tdurum_contig78972_316	Common reverse	CACCTTGACGCATCTCTCTA
	Allele 1	GAAGGTGACCAAGTTCATGCTCCGGTTTGACCGTGC
	Allele 2	GAAGGTCGGAGTCAACGGATTCCGGTTTGACCGTGT
BS00012068_51	Common reverse	ATGCCAAACATACATGACCA
	Allele 1	GAAGGTGACCAAGTTCATGCTAGACGTCGAAACTACCCAAT
	Allele 2	GAAGGTCGGAGTCAACGGATTAGACGTCGAAACTACCCAAC

4.3.7 High confidence genes within the QTLs in RefSeqv1.1

The physical position of the markers flanking the QTL in 1B were used to mark the QTL region (Figure 17). There were 70 high confidence genes within the 2.2 Mb region of short arm of chromosome 1B in the reference genome of Chinese Spring. The high confidence genes and expressed genes within the QTL regions and their physical positions are listed in Supplementary Table 5 and 6, respectively.

We also looked at differential gene expression between various tissue types in variety Chinese Spring reported by previously published RNA seq study (Yang et al., 2015). The RNA seq expression data was extracted from *wheat-expression.com*. Within the QTL interval, 32 genes were expressed in various tissue types. A heatmap of gene expression profile of these high confidence genes is given in Figures C1 and C2.

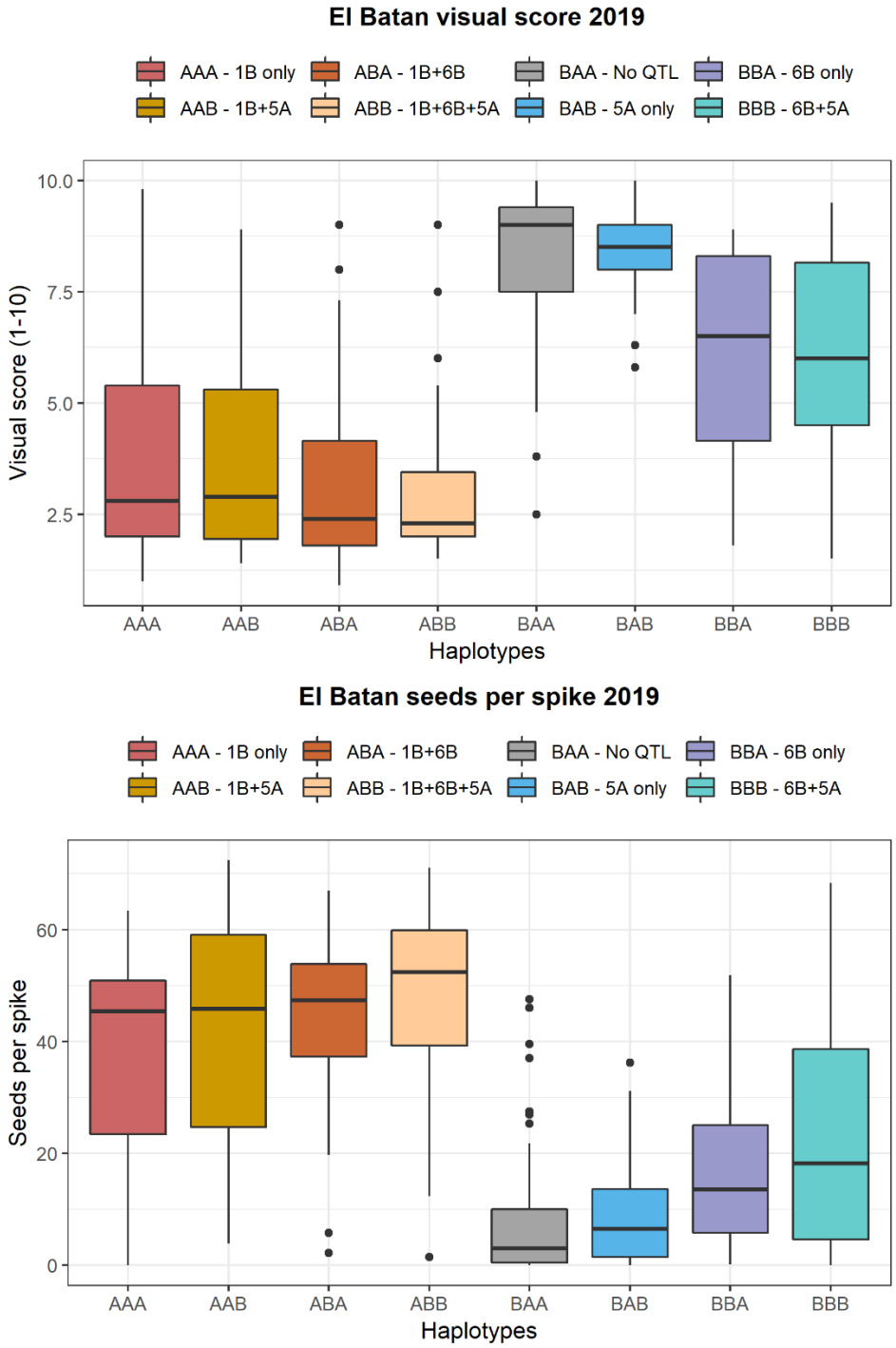

Figure 17 Additive effect of quantitative trait loci on sterility visual score (top panel) and average seed per spike (bottom panel) in El Batan test cross evaluation 2019.

4.3.8 Pentatricopeptide repeat (PPR) genes within the QTLs

Five genes of the PPR gene family (TraesCS1B02G038200, TraesCS1B02G038300, TraesCS1B02G038400, TraesCS1B02G038500 and TraesCS1B02G038600) were identified within the QTL interval in chromosome 1B in the reference genome of Chinese Spring (Table 12). These genes were found in a cluster within the QTLs interval. These genes have high sequence similarities with PPR genes from various grass species such as Brachypodium, Aegilops, Maize and Barley with more than 90% sequence identity. Top hits in BLAST results of these genes were predicted *Rf* proteins of mitochondrial origin with protein evidences in related grass species as *Aegilops*, *Brachypodium*, Oryza spp., *Panicum* and *Hordeum vulgare* (Figure C3).

4.3.9 Orthologs in other grass species

Orthologs of these genes were identified in sorghum (*Sorghum bicolor* L.) and Rice (*Oryza sativa* L. ssp. *japonica*) (Table 12). The sorghum ortholog SORBI_3006G273900 and rice orthologs (Os04g0351333, Os04g0349600, Os04g0350000) have a predicted *Rf* protein. BLAST search of sequences of these five genes against *Oryza sativa* Japonica group, gave hits on *Rf1*, *Rf4*, *Rf6* and *RF1b* genes in rice with very high sequence identity (91.9 – 94.6%).

Table 12 Pentatricopeptide repeats genes within the genomic regions associated with fertility restoration in cytoplasmic male sterile (CMS) wheat identified via linkage mapping in a recombinant inbred line population developed from a restorer source, Cargill 115. The genes are followed by their predicted annotations, homoeologues within the wheat genome and orthologs in other monocots where PPR genes have been known to restore fertility in CMS systems.

CHR	Gene ID (physical position)	Annotation	Homoeologues	Orthologs
1B	TraesCS1B02G038200 (17894194 -17896564 bp)	protein binding, ovule development, embryo development	TraesCS1D02G033000	Rice (Os04g0351333, Os04g0349600, Os04g0350000) SORBI_3006G273900,
1B	TraesCS1B02G038300 (17963319 – 17966241 bp)	protein binding	TraesCS1A02G031700, TraesCS1D02G032300	Rice (Os04g0351333, Os04g0349600, Os04g0350000)
1B	TraesCS1B02G038400 (18089772 – 18093637 bp)	protein binding	TraesCS1D02G033000	Rice (Os04g0351333, Os04g0349600, Os04g0350000)
1B	TraesCS1B02G038500 (18115008 – 18118673 bp)	protein binding		Rice (Os04g0351333, Os04g0349600, Os04g0350000)
1B	TraesCS1B02G038600 (18363377 – 18364565 bp)	protein binding	TraesCS6D02G018941	

4.4 Discussion

Cargill 115 can restore full fertility in a CMS cross as evidenced by the trait values in this experiment. Sterile florets per spike is the preferred trait over seeds per spike or visual score to assess if the restorer line can provide full restoration (Würschum et al., 2017). However, based on heritability estimates, visual score also appears to be a very reliable trait and can be successfully used for genetic mapping studies instead of resource and time intensive phenotyping for seed per spike and sterile florets. In the test cross evaluation, ~17% of the lines (n = 50) had trait values of zero sterile florets per spike. This data suggests that Cargill 115 has full fertility restoration capacity. In addition to Cargill 115, Cargill 116 and some RILs from this study can be used as restorer sources in hybrid wheat breeding. Cargill 116 is a restorer line that was genotyped with KASP markers developed and it has the same marker haplotype as Cargill 115.

In RIL per se evaluation of El Batan experiment and test cross evaluation in 2018, a minor QTL in chromosome 5A was identified which maps to the distal end of chromosome 5AL. Several previous studies using monosomic analysis have identified minor effect genes in 5A (Bahl and Maan, 1973; Maan, 1985). These minor effect genes are known to be inconsistent in expression (Bahl and Maan, 1973; Miller et al., 1974) due to environmental effects and they might have failed to express or have a low expression in our experiments which was harder to detect in presence of large effect QTLs in the test cross evaluations. Other interesting observation is that this QTL was detected only in El Batan in two experiments; RILs phenotyping and test cross evaluation. This could be due to environment dependent expression pattern in fertility restoration genes as have been previously identified by other researchers (Bahl and Maan, 1973).

The major effect QTL in 1B is most likely fertility restoration gene *Rf3* reported previously by various studies using different restorer sources (Ahmed et al., 2001; Geyer et al., 2016; Kojima et al., 1997; Livers, 1964; Ma and Sorrells, 1995; Tahir and Tsunewaki, 1969; Würschum et al., 2017; Yen et al., 1969; Zhou et al., 2005). Geyer et al (2016) identified a SNP marker IWB72107 (synonym = Tdurum_contig50667_306) mapped in chromosome 1BS at 20.58 Mb in the RefSeqv1.1 which is tightly linked with Rf3 in line R113. R113 is the restorer line in which Rf3 was first identified by Maan (1985). The SNP that is closest to the peak in our study is Tdurum_contig78972_316 which is physically located at 18.42 Mb in chromosome 1BS. These markers are close enough to assume that the major effect QTL in 1B is *Rf3*. Tdurum_contig78972_316 has been converted into user-friendly KASP marker, validated in this RIL and independent population. This KASP marker can be used for marker-assisted selection in breeding programs. Wurshum et al (2017) also mapped *Rf3* using GBS markers to chromosome 1BS. A direct comparison between this study and Wurshum et al (2017) could not be made because they used a genotyping by sequencing approach for genotyping and physical positions were not reported in that study.

Fertility restorer genes in chromosome 6B have been reported before using monosomic analysis and is referred to as *Rf4* (Bahl and Maan, 1973; Du et al., 1991; Maan, 1985). The *Rf4* gene was identified in R113 by Maan in 1985 (Maan, 1985). The same line was used as a restorer source in a BC1F1 population developed by Geyer et al. (2018) and they mapped Rf4 to 6B. The closest marker to Rf4 in their study was IWA52, the physical position of which is at 32.33 Mb in chromosome 6BS in Chinese spring. The confidence interval reported for *Rf4* in 6B in the Geyer et al. (2018) was large, spanning 25 cM. We extracted the physical position of markers within 25 cM of marker IWA52 in the linkage map used by Geyer et al. (2018) which is

the entire short arm of chromosome 6B. The QTL peaks identified in this study in chromosome 6B is at 4.87 and 20.71 Mb. The QTL interval identified on 6BS in this study is 4 – 34 Mb in Chinese Spring, which is also a very large genomic region. The region identified in this study overlaps with that identified by Geyer et al. (2018) in R113, indicating that the QTL identified in this study in 6B might be *Rf4*. The phenotypic effect of this gene estimated in these previous studies is modest. The phenotypic variance explained by this QTL in this study is consistent but relatively low at 7.5 to 9.2 % phenotypic variance. Previous studies done in line R113 and populations developed using this line as a restorer source have reported a low positive effect on fertility restoration but a cumulative negative effect when interacting with *Rf1* (Geyer et al., 2018; Maan, 1985).

The QTL regions identified in this study is relatively large (~2.2 Mb) since this is a linkage mapping study with a population size of 299. There are 70 high confidence annotated genes within this region in Chinese Spring reference genome (RefSeq 1.1). By looking at the RNA seq expression data extracted from public databases, the numbers were narrowed down to 37. CMS in plants is a manifestation of chimeric mitochondrial open reading frames (ORFs). The expressed proteins from these ORFs interfere with functional pollen formation by premature degeneration of the tapetum layer in anthers during pollen formation (Schnable and Wise, 1998). The tapetum is a layer that surrounds the developing pollen grains in microsporangium which provides them with nutrients during their development. Nuclear restorers often interfere with the expression of these chimeric mitochondrial proteins and suppress the deleterious effects associated with them in anthers (Schnable and Wise, 1998). In light of this information, it is logical to look at the expression of genes in normal and deformed anthers. Fortunately, RNA sequencing data of normal stamens and its pistillody mutant in Chinese Spring is previously

published and publicly available (Yang et al., 2015). We extracted the expression data from these two different tissue types of Chinese Spring and identified seven differentially expressed genes within the QTL interval. Most of the differentially expressed genes had functions as “protein binding”, “serine-type endopeptidase inhibitor activity” and “integral component of membranes”. Since *Rf* genes have been previously reported in Chinese Spring, these genes could be candidate genes for fertility restoration.

Another approach that was taken to identify candidate genes was looking at the PPR gene families. PPR gene products are RNA binding proteins that have post-translational functions inside mitochondria and chloroplasts in RNA editing, splicing, cleavage and translation (Schmitz-Linneweber and Small, 2008). CMS originates from the mitochondria in plant cell and these PPR genes operate in post-translational functions inside mitochondria, hence they appear very important for fertility restoration. PPR gene family has been implicated for fertility restoration in many modern hybrid crop species that use CMS for hybridization such as sorghum (Klein et al., 2005), rice (Wang et al., 2006), canola (Brown et al., 2003), radish (Liu et al., 2012) peppers (Min et al., 2008) and petunia (Bentolila et al., 2002). PPR genes occur in clusters in all plant genomes (Schmitz-Linneweber and Small, 2008) and screening for PPR genes in QTL regions of Refseq1.1 identified five PPR genes within the QTL confidence interval in 1B (Table 4). All of these five genes have more than 90% sequence similarity with Rf1 like proteins in several model grass species and rice. These genes also have orthologs in sorghum and rice which have predicted functions of fertility restoration. These genes are good candidates for fertility restoration.

4.5 Conclusions

Cargill 115 wheat has full fertility restoration capacity in CMS wheat of *Triticum timopheevi* origin. Fertility restoration genetics in Cargill 115 is very complex and is conditioned by *Rf3*, *Rf4* and an additional minor effect QTL in 5A. KASP markers developed from tightly linked markers to these genes and QTL can be used for MAS in hybrid wheat. The PPR genes within the QTL confidence intervals are good candidates for fertility restoration.

CHAPTER V

CONCLUSIONS AND FUTURE RESEARCH

Hybrid wheat offers great promise in terms of grain yield and commercial heterosis. There is sufficient high-parent and commercial heterosis for hybrid wheat to be commercially viable in Texas. Moreover, genetic gains can be made for heterosis via use of elite lines from two breeding programs serving different geographical locations and growing conditions; since most of the heterosis is a result of general combining ability (GCA). It can also be concluded that use of (chemical hybridizing agents) CHAs for hybrid seed production is a reliable method since it does not impact grain yield of the resultant hybrids.

GCA can be very good predictor of hybrid yield. Selection of parents for making single crosses can be made by use of GCA estimates since it predicts hybrid yield in wheat with high accuracy. Absence of specific combining ability (SCA) variance indicates that development of heterotic pools is crucial for exploitation of dominance effects in hybridization.

Future research efforts need to be focused on developing heterotic pools based on GCA estimates and other traits necessary for hybridization. Reciprocal recurrent selection schemes would be needed to make improvements within the pools, which is already happening as line breeding programs of Texas A&M University and University of Nebraska, Lincoln continue research in developing new pureline cultivars. A more prudent strategy would be to assign each program the sole responsibility of developing either male or female lines. This would make reciprocal recurrent improvement more practical and fit well within the current line breeding efforts of each program. In addition, future research needs to continue on evaluation of new promising lines for floral traits and combining abilities. The lines then should be assigned to

male or female pools based on their floral traits, combining ability estimates and genetic similarities.

Due to the ease of turning any high yielding line into female parent, CHA is most likely going to be the method of choice for hybrid seed production at early stages of the breeding cycle. Moreover, high number of crosses need to be made at early stages. This makes hybrid seed production a cost prohibitive step in the breeding pipeline. As the data from this study suggests, F_2 testing can be effectively utilized to support decision making in selection of F_1 hybrids for advanced yield trials. Hybrid wheat lines with positive midparent and high parent heterosis at F_2 stage are very safe candidates for further evaluations in a hybrid wheat-breeding program.

The F_1 versus F_2 comparison made as a part of the study described in chapter III of this dissertation involved lines with wide range of grain yield. However, if F_2 data would be used for supplementing selection decisions, it would make no sense to include low yielding hybrid lines in the F_2 trial. We would only want further evaluation of promising hybrid lines in the F_1 evaluation. The relationship between F_1 and F_2 heterosis is more promising in high yielding hybrid crosses as compared to low yielding ones, based on the data presented in chapter III. This leads to the conclusion that further studies should include F_2 evaluation of only high yielding crosses. Moreover, optimizing F_2 evaluation sites is needed to take care of G×E interaction and ultimately use F_2 data with confidence for making selection decisions.

Full fertility restoration in a CMS background of *Triticum timopheevi* origin is possible via use of Cargill 115 as a restorer source or a male line. Based on the extensive phenotyping and linkage mapping work conducted as a part of the dissertation research work in chapter III, fertility restoration genetics in Cargill 115 is very complex and is conditioned by *Rf3*, *Rf4* and an additional minor effect QTL in 5A. KASP markers developed from tightly linked markers to

these genes and QTL can be used for marker assisted selection in hybrid wheat. The PPR genes within the QTL confidence intervals are good candidate for fertility restoration.

Future research should be directed towards studying the effect of these restorer genes in different backgrounds. The restorer genes need to be transferred to other male line and fertility restoration needs to be studied. In addition, fine mapping efforts need to be carried out in effort to better map *Rf4* and study its effect in absence of *Rf3*. Molecular markers that are tightly linked to *Rf4* need to be identified so that *Rf3* and *Rf4* can be easily used in breeding superior male lines for CMS based hybrid seed production.

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APPENDIX A

Table A1. Model fit summary of spatial models tested in experiment two planted in Bushland and Greenville in 2017 and their combined analysis. The models selected to extract yield estimates for further analysis are in bold and underlined.

	Bushland		**Greenville**		**Combined**	
	AIC	**-log**	**AIC**	**-log**	**AIC**	**-log**
					Heterogeneous errors	
Incomplete block	7407.30	-3700.65	7837.81	-3915.90	15369.13	-7679.56
Incomplete block + row	7409.30	-3700.65	7839.63	-3915.81	15372.68	-7679.34
Incomplete block + column	**7395.59**	**-3693.79**	7839.67	-3915.83	15360.09	-7673.04
Incomplete block + row + column	7397.59	-3693.79	7841.49	-3915.74	15363.64	-7672.82
AR1 (row)	7397.97	-3695.99	**7837.59**	**-3915.79**	**15324.18**	**-7656.09**
Exponential (row)	7397.97	-3695.99	7837.59	-3915.79	15324.18	-7656.09
Gaussian (row)	7399.69	-3696.85	7837.62	-3915.81	15327.61	-7657.80
AR1 (column)	7412.70	-3703.35	7837.68	-3915.84	15341.39	-7664.70
Exponential (column)	7412.70	-3703.35	7837.68	-3915.84	15341.39	-7664.70
Gaussian (column)	7413.17	-3703.59	7837.65	-3915.82	15342.09	-7665.04
AR1 (row) x AR1 (column)	7399.00	-3695.50	7839.46	-3915.73	15326.32	-7655.16
Incomplete block + AR1 (row)	-	-	7839.585	-3915.79	15349.44	-7667.72
Incomplete block + column + AR1(row)	-	-	7841.525	-7841.53	15346.02	-7665.01
					Homogeneous errors	
Incomplete block					15787.83	-7888.913
Incomplete block + row					15789.83	-7888.913
Incomplete block + column					15783.26	-7885.63
Incomplete block + row + column					15785.26	-7885.63
AR1 (row) x AR1 (column)					-7843.483	-7843.483

Table A2. Parental combination which have significant differences between cross and reciprocal cross as evidenced by paired t-tests in experiment 1 planted in 2016 and experiment 2 planted in 2017.

Year	Cross	Means		ΨDifference	Cross direction
		Cross	Reciprocal cross		
2016	ROBIDOUX / TX12M4063	1936.38	3280.41	1344.03	NE × TX
2016	TX10D2230 / TX11D3026	1921.39	3141.14	1219.75	TX × TX
2016	TX09D1172 / TX12M4063	2249.26	3395.00	1145.73	TX × TX
2016	FREEMAN / TX12M4004	2125.56	3112.93	987.36	NE × TX
2016	TX10D2230 / TX12M4065	2316.81	3063.37	746.56	TX × TX
2016	TX11D3008 / TX12M4063	2209.27	2857.03	647.76	TX × TX
2016	TX11D3049 / TX12M4063	2414.81	3055.13	640.32	TX × TX
2016	TX11D3008 / TX11D3112	2257.06	2896.08	639.02	TX × TX
2016	LCH13NEDH-11-24 / NE10589	675.82	1298.77	622.96	NE × NE
2016	NE07531 / TX09D1172	722.23	1276.00	553.78	NE × TX
2016	NE07531 / WESLEY	671.05	1216.41	545.35	NE × NE
2016	TX11D3129 / TX12M4063	2774.97	3311.15	536.18	TX × TX
2016	TX10D2363 / TX11D3112	2485.86	3018.03	532.17	TX × TX
2016	NE10683 / PSB13NEDH-15-58W	953.02	1435.91	482.89	NE × NE
2016	LCH13NEDH-11-24 / OVERLAND	985.32	1454.17	468.85	NE × NE
2016	TX10D2230 / TX12M4063	2593.85	3028.09	434.25	TX × TX
2016	TX11D3129 / TX12M4065	2781.12	2371.56	409.56	TX × TX
2016	NE07531 / SETTLERCL	1054.40	1462.02	407.62	NE × NE
2016	NE07531 / PANHANDLE	913.83	1315.09	401.27	NE × NE
2016	NE07531 / NE10683	1286.66	890.08	396.58	NE × NE
2016	TX10D2063 / TX10D2230	2458.29	2853.29	395.00	TX × TX
2016	LCH13NEDH-11-24 / WESLEY	1065.07	1430.79	365.73	NE × NE

Table A2 continued

Year	Cross	Means		ΨDifference	Cross direction
		Cross	Reciprocal cross		
2016	OVERLAND / WESLEY	1450.63	1088.07	362.55	NE × NE
2016	LCH13NEDH-11-24 / NE10683	908.98	1265.37	356.38	NE × NE
2016	NE10683 / PANHANDLE	1082.84	1432.36	349.52	NE × NE
2016	TX12M4063 / TX12M4065	2506.89	2847.36	340.47	TX × TX
2016	GOODSTREAK / NE07531	1354.88	1059.07	295.81	NE × NE
2016	TX10D2230 / TX11D3112	2864.45	2569.59	294.85	TX × TX
2016	NE10683 / WESLEY	1097.17	1392.00	294.83	NE × NE
2016	NE10683 / ROBIDOUX	1325.45	1054.12	271.34	NE × NE
2016	TX11D3026 / TX12M4065	2748.00	2492.78	255.22	TX × TX
2016	TX12M4004 / TX12M4065	3142.87	2890.33	252.54	TX × TX
2016	NE10589 / PANHANDLE	1129.37	1381.63	252.26	NE × NE
2016	TX10D2363 / TX12M4063	2735.76	2488.51	247.25	TX × TX
2016	LCH13NEDH-11-24 / PSB13NEDH-15-58W	1021.98	1246.53	224.55	NE × NE
2016	TX11D3112 / TX12M4004	2646.30	2436.05	210.25	TX × TX
2016	TX10D2063 / TX12M4063	2619.87	2419.69	200.18	TX × TX
2016	LCH13NEDH-11-24 / ROBIDOUX	1100.47	1298.56	198.09	NE × NE
2016	NE10683 / SETTLERCL	1057.40	1242.65	185.26	TX × TX
2016	FREEMAN / NE07531	1205.10	1378.10	173.00	NE × NE
2016	TX11D3112 / TX12M4063	2708.46	2575.35	133.12	TX × TX
2016	TX11D3112 / TX12M4065	3024.54	3155.29	130.75	TX × TX
2016	TX10D2063 / TX12M4065	2641.96	2755.03	113.08	TX × TX
2016	PANHANDLE / WESLEY	1246.55	1316.45	69.90	NE × NE
2016	LCH13NEDH-11-24 / NE07531	1132.32	1103.10	29.22	NE × NE

Table A2 continued

Year	Cross	Means		ΨDifference	Cross direction
		Cross	Reciprocal cross		
2016	TX10D2363 / TX12M4065	2957.77	2977.52	19.75	TX × TX
2017	NE10589 / NE07531	2580.37	2660.62	80.25	NE × NE
2017	PANHANDLE / NE07531	2070.16	2287.11	216.95	NE × NE
2017	NE10589 / TX11D3112	2521.12	2646.23	125.11	NE × TX
2017	TX11D3049 / TX11D3112	2207.19	2238.54	31.35	TX × TX
2017	TX10D2363 / TX11D3112	2426.34	2776.11	349.77	TX × TX
2017	FREEMAN / TX11D3049	1948.86	2295.36	346.51	NE × TX
2017	NE10589 / GOODSTREAK	2213.25	2231.01	17.76	NE × NE
2017	ROBIDOUX / GOODSTREAK	2161.22	2302.15	140.93	NE × NE
2017	PSB13NEDH-15-58W / GOODSTREAK	2139.05	2318.75	179.70	NE × NE
2017	TX12M4063 / TX09D1172	2509.35	2620.18	110.83	TX × TX
2017	PANHANDLE / TX09D1172	2267.54	2273.23	5.69	NE × TX
2017	PSB13NEDH-15-58W / TX09D1172	2483.01	2754.75	271.75	NE × TX
2017	NE10589 / TX09D1172	2636.42	2671.69	35.28	NE × TX
2017	FREEMAN / TX09D1172	2613.00	2705.81	92.81	TX × TX
2017	TX12M4065 / FREEMAN	2478.58	2780.46	301.87	TX × NE
2017	TX11D3129 / FREEMAN	2455.37	2693.88	238.51	TX × NE
2017	PANHANDLE / TX10D2230	2524.59	2674.86	150.27	NE × TX
2017	FREEMAN / WESLEY	2144.36	2232.97	88.61	NE × NE
2017	TX12M4063 / WESLEY	2610.60	2636.16	25.56	TX × NE
2017	TX10D2063 / TX10D2363	2539.18	2793.86	254.69	TX × TX
2017	TX09D1172 / TX10D2363	2535.30	2580.16	44.86	TX × TX
2017	NE10683 / PANHANDLE	2175.76	2383.21	207.45	NE × NE

Table A2 continued

Year	Cross	Means		ΨDifference	Cross direction
		Cross	Reciprocal cross		
2017	OVERLAND / ROBIDOUX	2538.30	2721.37	183.07	NE × NE
2017	FREEMAN / ROBIDOUX	2538.25	2612.25	73.99	NE × NE
2017	TX11D3129 / ROBIDOUX	2349.87	2805.12	455.24	TX × NE
2017	WESLEY / TX12M4065	2542.07	2579.53	37.46	NE × TX
2017	TX12M4065 / PSB13NEDH-15-58W	2565.91	2608.84	42.93	TX × NE
2017	NE10683 / PSB13NEDH-15-58W	2031.07	2295.47	264.40	NE × NE
2017	NE07531 / PSB13NEDH-15-58W	2262.18	2284.17	21.99	NE × NE
2017	TX11D3129 / TX12M4063	2619.00	2747.49	128.49	TX × TX
2017	TX12M4063 / OVERLAND	2524.04	2605.97	81.93	TX × NE
2017	PANHANDLE / OVERLAND	2449.87	2749.64	299.78	NE × NE
2017	TX12M4065 / NE10683	2573.29	2619.47	46.18	TX × NE
2017	TX11D3112 / NE10683	2239.93	2246.16	6.23	TX × NE
2017	NE07531 / TX12M4004	2563.78	2618.46	54.68	NE × TX
2017	HARRY / TX11D3129	2025.57	2160.36	134.79	NE × TX
2017	HARRY / ROBIDOUX	2257.59	2310.39	52.80	NE × NE
2017	HARRY / TX10D2230	2203.00	2350.32	147.32	NE × TX
2017	WESLEY / HARRY	2211.36	2287.72	76.36	NE × NE
2017	PANHANDLE / HARRY	1855.43	2268.33	412.90	NE × NE
2017	NE10683 / HARRY	2210.72	2269.47	58.75	NE × NE
2017	HARRY / GOODSTREAK	1984.61	2358.92	374.31	NE × NE

ΨAbsolute value of the difference between cross means and reciprocal cross means

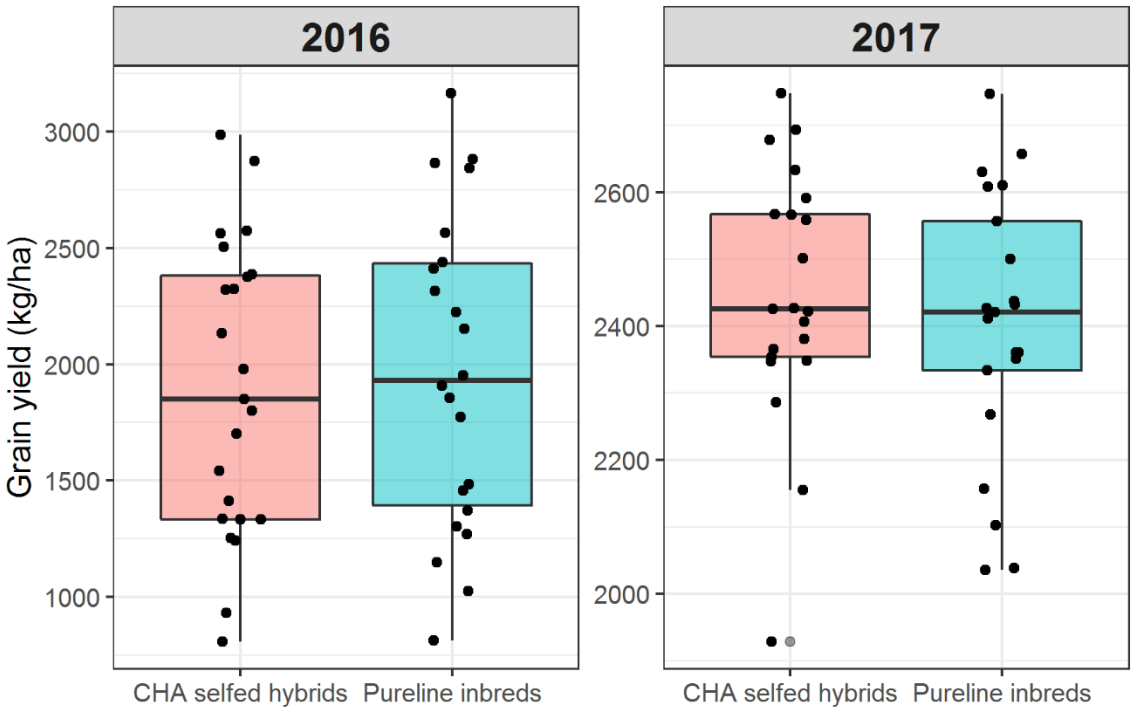

Figure A1. Boxplots showing effects of chemical hybridizing agent on hybrid wheat grain yield in experiment 1 which was planted at McGregor, Texas in 2016 and experiment 2 which was planted at Greenville and Bushland, Texas in 2017. Boxes labeled CHA selfed hybrids refer to genotypes that were selfed by crossing the same genotypes using CHA whereas boxes labeled pureline inbreds represents pureline inbreds that were included in the experiments as checks or parents.

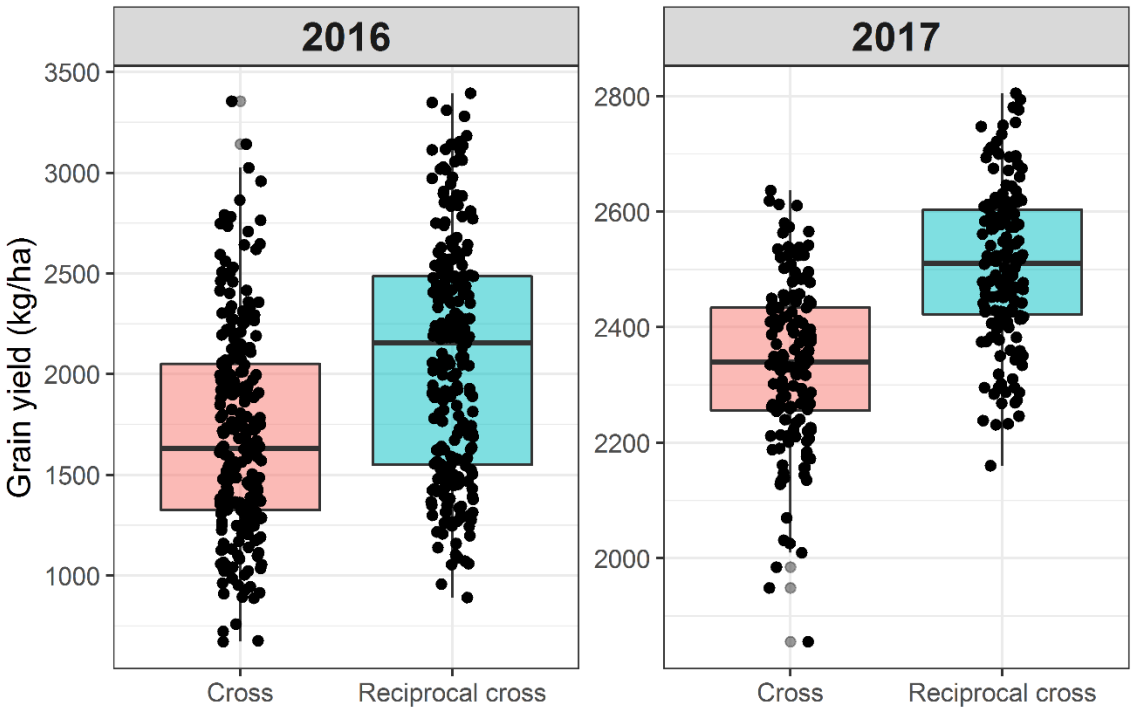

Figure A2. Boxplots showing hybrid wheat grain yield in combinations of parents as crosses and reciprocal crosses from experiment 1 which was planted at McGregor, Texas in 2016 and experiment 2 which was planted at Greenville and Bushland, Texas in 2017.

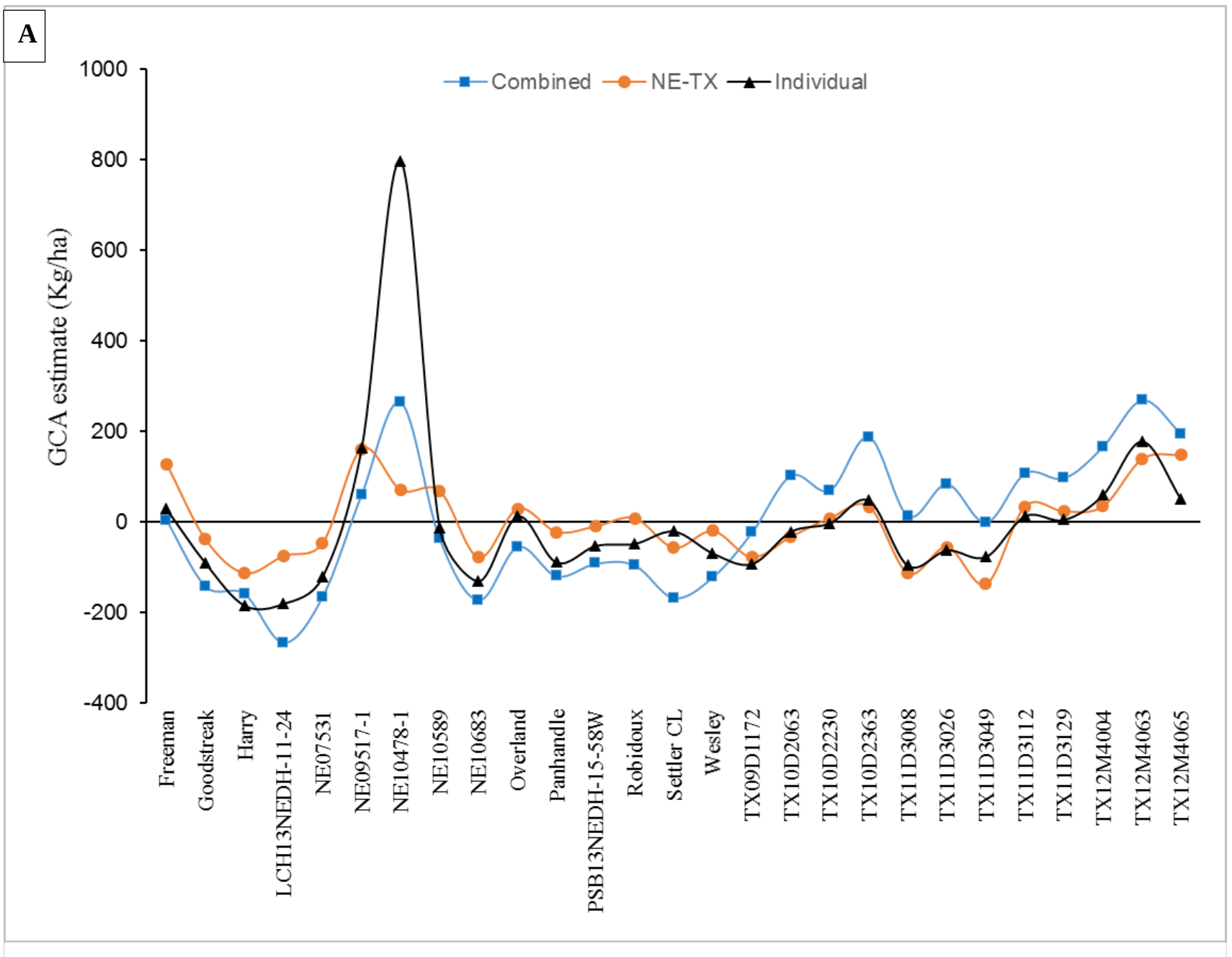

Figure A3. Four analyses were conducted for combining ability estimation using the yield data from two hybrid wheat field experiments planted in 2016 and 2017 in Texas. The first was the full diallel analysis involving all the data; the second was analysis of hybrid crosses involving Texas parents only (TX subset), the third was the analysis of hybrid crosses with Nebraska parents only (NE subset) and fourth was the subset of hybrids of Texas by Nebraska crosses or vice versa only (mixed subset). **A)** Line graph representing general combining ability (GCA) estimates from a full diallel dataset analysis and analysis of the three subsets of the full dataset. **B)** Bar graphs representing GCA estimates and their corresponding standard errors from the full diallel analysis and the three subsets. The Combined line/bar represents GCA estimates from the full diallel analysis. The NE-TX line/bar represents GCA estimates from analysis of mixed subset only. The Individual line/bar represents GCA estimates of TX lines from analysis of TX subset and GCA estimates of NE lines from analysis of NE subset.

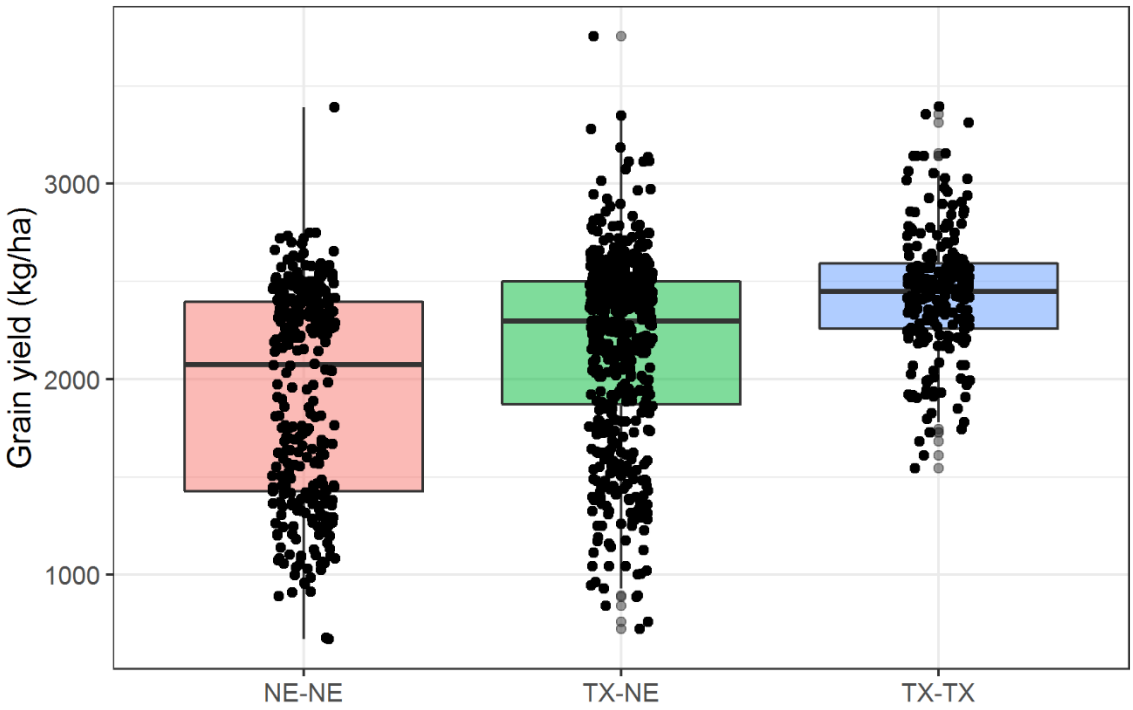

Figure A4. A boxplot of hybrid yield from two hybrid wheat field experiments planted in 2016 and 2017 in Texas. The NE-NE box represents yield ($kgha^{-1}$) from hybrid crosses involving parents from Nebraska only, the TX-NE box represents hybrids yield ($kgha^{-1}$) from hybrid crosses involving parents from Nebraska and Texas and the third box represents hybrids yield ($kgha^{-1}$) from hybrid crosses involving parents from Texas only

APPENDIX B

Table B1. Hybrid and pureline entries included in F_2 evaluation experiments in 2017 and 2018 with their pedigrees.

Entry name	Class	Pedigree (Female/Male)	Experiments
NXB15-6502	Hybrid	TX12M4063/TX09D1172	2017, 2018*
NXB15-6699	Hybrid	TX12M4063/PANHANDLE	2017, 2018
NXB15-6955	Hybrid	TX12M4063/ROBIDOUX	2017, 2018
NXB15-7175	Hybrid	TX12M4004/TX12M4063	2017, 2018
NXB15-7310	Hybrid	TX12M4063/TX11D3129	2017, 2018
NXB15-6534	Hybrid	FREEMAN/TX09D1172	2017, 2018
NXB15-6563	Hybrid	PSB13NEDH-15-58W/FREEMAN	2017, 2018
NXB15-6567	Hybrid	ROBIDOUX/FREEMAN	2017, 2018
NXB15-6583	Hybrid	TX09D1172/FREEMAN	2017, 2018
NXB15-6822	Hybrid	TX11D3008/WESLEY	2017, 2018
NXB15-6931	Hybrid	FREEMAN/ROBIDOUX	2017, 2018
NXB15-7126	Hybrid	FREEMAN/PSB13NEDH-15-58W	2017, 2018
NXB15-7230	Hybrid	FREEMAN/OVERLAND	2017, 2018
NXB15-7478	Hybrid	WESLEY/LCH13NEDH-11-24	2017
NXB15-7486	Hybrid	NE09517-1/LCH13NEDH-11-24	2017
NXB15-6110	Hybrid	TX12M4065/TX11D3112	2017
NXB15-6979	Hybrid	TX12M4063/NE09517-1	2017
NXB15-7054	Hybrid	TX11D3112/TX12M4065	2017
NXB15-7455	Hybrid	TX10D2230/LCH13NEDH-11-24	2017
NXB15-7462	Hybrid	TX12M4065/LCH13NEDH-11-24	2017
NXB15-6426	Hybrid	WESLEY/TX11D3008	2017
NXB15-6546	Hybrid	NE10478-1/FREEMAN	2017
NXB15-7015	Hybrid	LCH13NEDH-11-24/NE09517-1	2017
NXB15-6162	Hybrid	PANHANDLE/NE10589	2018
NXB15-6759	Hybrid	OVERLAND/TX10D2230	2018
NXB15-6754	Hybrid	WESLEY/TX10D2230	2018
NXB15-7298	Hybrid	TX10D2363/TX11D3129	2018
NXB15-6163	Hybrid	OVERLAND/NE10589	2018
NXB15-7295	Hybrid	TX09D1172/TX11D3129	2018
NXB15_7426	Hybrid	TX10D2230/TX12M4004	2018
NXB15-6950	Hybrid	TX12M4065/ROBIDOUX	2018
NXB15-7186	Hybrid	TX10D2363/TX12M4063	2018
NXB15-7222	Hybrid	TX12M4063/OVERLAND	2018
OVERLAND	Pureline		2017, 2018
PANHANDLE	Pureline		2017, 2018
PSB13NEDH-15-58W	Pureline		2017, 2018

Table B1 continued

Entry name	Class	Pedigree (Female/Male)	Experiments
FREEMAN	Pureline		2017, 2018
ROBIDOUX	Pureline		2017, 2018
TX09D1172	Pureline		2017, 2018
TX10D2230	Pureline		2017, 2018
TX11D3008	Pureline		2017, 2018
TX11D3129	Pureline		2017, 2018
TX12M4004	Pureline		2017, 2018
TX12M4063	Pureline		2017, 2018
TX12M4065	Pureline		2017, 2018
WESLEY	Pureline		2017, 2018
TX11D3112	Pureline		2017
LCH13NEDH-11-24	Pureline		2017
NE09517-1	Pureline		2017
NE10478-1	Pureline		2017
TX10D2363	Pureline		2018
TX10D2063	Pureline		2018
RUTH	Pureline		2018
NE10589	Pureline		2018

*Entries repeated in both experiments (2017 and 2018)

Table B2. Mean squares from analysis of variance of field experiments from single locations and combined analysis across locations in 2017 and 2018.

2017							
Source of Variation	**Bushland**	**Chillicothe**	**Grant**	**Lincoln**	**Greenville**	**McGregor**	**Combined**
Blocks	48108.63	724010.42***	1145639.59***	246536.97	622184.66	874105.01***	730647.9***
Iblocks	218178.53**	117530.43***	362394.68***	831438.62***	278126.75	47999.59	229169.7
Genotypes	308989.38***	134322.04***	234784.81**	839169.91***	692858.01***	1364217.15***	945151.7***
Environment							181687485.6***
G × E							666253.5***
Error	75565.72	31595.45	102913.64	173126.59	273385.13	60485.95	134048
2018							
Blocks	109200.613			238386.91	1405557.93**	64215.7	488907.6
Iblocks	298026.873***			307514.6***	365413.82**	509123.09	258772.1
Genotypes	81529.512*			501731.58***	272632.25*	475102.43*	390317.4**
Environment							296049713.41***
G × E							398999.41***
Error	60485.95			76897.29	154237.25	401758.86	204400

*** Significant at ($P \leq 0.0001$)

** Significant at ($P \leq 0.001$)

Table B3. Mean squares from analysis of variance of field experiments from combined analysis across locations in 2017 and 2018. The locations were grouped according to results from a GGE Biplot analysis.

Source of Variation	**Group I (BD, CH, MCG)**	**Group II (LN, GR)**	**Group I (LN, MCG)**
	2017		***2018***
Blocks	426973.8***	409495.9	45832.4
Iblocks	89638.1	229581.4	275484
Genotypes	952400.6***	762285.1***	572607.3**
Environment	123198000.4***	253774774***	162654540.7***
G × E	537421.9***	483428.1***	452622.6*
Error	61972.9	173367.1	247775.4
R^2	0.96	0.93	0.86
CV	12.58	11.44	11.01
Grand Mean	1978.57	3638.95	4520.38

Location abbreviations - BD: Bushland, CH: Chillicothe, MCG: McGregor, LN: Lincoln, GR: Grant

Table B4. Hybrid entries in 2017 F_2 experiment with their respective mid-parent (MPH) and high-parent heterosis (HPH) estimates calculated from single locations, locations groups together according to GGE biplot analysis and all locations combined. The F_1 column indicates heterosis estimates of the same genotypes at F_1 stage extracted from a previously published F_1 evaluation study (Adhikari et al. 2020. 2020).

	BD		CH		Grant		Lincoln		McGregor		Greenville		TX group		NE group		Combined		F_1	
Hybrid entries	**MPH**	**HPH**	**MPH**	**HPH**	**MPH**	**HPH**	**MPH**	**HPH**	**MPH**	**HPH**	**MPH**	**HPH**	**MPH**	**HPH**	**MPH**	**HPH**	**MPH**	**HPH**	**MPH**	**HPH**
NXB15-6110	16.66	11.80	40.68	20.17	0.70	-15.49	-9.63	-13.21	420.55	222.03	75.59	73.94	54.81	39.19	-5.70	-10.90	27.52	17.85	8.47	5.06
NXB15-6426	-14.01	-20.98	-22.70	-24.26	-12.37	-13.60	-11.52	-16.20	-86.44	-86.91	-7.61	-14.78	-34.35	-37.77	-14.71	-18.01	-20.97	-21.07	-62.85	-65.06
NXB15-6502	-6.26	-13.86	-1.62	-3.61	-12.30	-13.53	0.92	-4.42	-0.17	-3.63	19.89	10.59	-2.78	-7.85	-2.11	-5.90	0.23	0.10	21.81	17.95
NXB15-6534	22.88	6.62	-12.66	-15.94	27.55	25.90	32.86	26.71	-73.31	-84.04	-20.39	-30.07	-8.39	-15.83	33.08	29.84	5.25	-1.46	0.34	-8.40
NXB15-6546	9.08	5.81	11.70	4.07	31.67	29.55	20.97	16.55	-39.31	-60.75	14.23	3.74	-4.42	-15.60	28.66	25.56	12.77	7.63	-10.43	-11.23
NXB15-6563	-6.89	-7.69	15.30	10.90	6.85	-2.26	-10.62	-17.91	-12.91	-31.62	-7.86	-10.33	2.53	-3.33	-1.10	-10.58	-1.13	-4.69	-16.06	-26.66
NXB15-6567	0.33	-2.27	-6.42	-16.46	-8.43	-9.43	1.53	-0.01	-65.68	-77.25	-30.54	-32.05	-11.97	-22.20	-3.73	-4.06	-10.03	-13.16	-4.00	-14.24
NXB15-6583	21.01	14.30	7.32	3.72	-19.12	-20.17	-5.65	-9.76	85.03	83.93	25.36	16.58	27.90	25.13	-14.23	-16.51	5.43	2.09	0.41	-8.32
NXB15-6699	6.25	0.35	42.10	37.33	-24.93	-25.91	-3.37	-7.58	457.49	454.17	31.17	21.98	57.89	54.46	-14.61	-16.88	14.03	10.43	20.71	16.37
NXB15-6822	-25.36	-26.21	15.47	11.53	-7.78	-9.41	-29.79	-34.74	479.83	315.66	46.69	11.26	16.98	16.44	-23.86	-26.26	-3.73	-5.35	9.22	7.98
NXB15-6931	-4.86	-5.67	12.86	8.56	9.17	-0.14	5.52	-3.09	-34.79	-48.80	-1.65	-4.28	-0.43	-6.12	6.78	-3.47	4.30	0.54	2.14	-7.50
NXB15-6955	10.30	7.28	23.17	21.72	1.72	-0.44	-5.43	-5.55	15.12	-4.74	15.92	0.85	15.09	9.07	-2.35	-3.36	8.54	8.05	20.64	15.86
NXB15-6979	6.01	2.14	30.33	19.62	-14.30	-15.99	0.40	-13.40	423.75	410.29	-7.79	-8.22	47.79	45.01	-6.46	-15.55	11.93	6.46	36.86	34.89
NXB15-7015	12.71	8.16	19.13	0.83	3.00	-1.66	3.86	-11.32	-78.95	-85.87	26.52	22.95	-10.11	-25.00	-0.25	-7.34	-1.31	-11.76	-76.52	-93.54
NXB15-7054	29.34	16.05	27.91	19.20	18.46	4.49	4.66	-0.07	-13.34	-20.38	13.41	-13.78	16.70	6.66	7.03	-0.33	13.65	12.94	-0.34	-4.07
NXB15-7126	19.24	3.46	-13.42	-16.68	8.09	6.69	48.77	41.88	-72.20	-83.37	-33.74	-41.80	-11.34	-18.54	30.11	26.94	0.06	-6.31	4.13	-4.63
NXB15-7175	5.26	-0.21	-8.97	-30.03	10.53	2.97	-11.22	-15.16	78.43	-3.08	-5.48	-30.40	11.67	-6.80	-5.54	-11.59	2.65	-12.56	11.44	5.15
NXB15-7230	-1.18	-2.30	7.57	3.90	-8.65	-10.26	-22.73	-28.17	39.50	0.00	25.19	-5.05	3.82	3.35	-18.49	-21.06	-6.72	-8.29	-10.40	-14.70
NXB15-7310	-11.07	-16.71	9.73	-14.53	-0.36	-12.21	-12.50	-18.49	87.33	0.88	53.76	36.13	11.89	-10.78	-8.20	-8.29	7.98	-4.60	23.86	22.39
NXB15-7455	-8.70	-10.76	23.16	1.18	-12.34	-20.57	23.93	2.61	473.28	300.00	7.45	-13.47	29.43	21.62	10.67	1.25	18.40	14.66	17.30	9.29
NXB15-7462	16.17	11.32	47.88	26.32	1.90	-14.47	-13.46	-16.88	371.78	191.86	92.04	90.24	49.05	34.01	-6.41	-11.57	24.83	15.36	26.85	16.97
NXB15-7478	5.05	3.93	0.94	-6.32	1.07	-7.42	-11.31	-21.26	-74.79	-86.48	46.94	22.62	-14.46	-28.47	-5.18	-14.87	-2.93	-5.52	-43.36	-45.96
NXB15-7486	8.73	0.69	5.13	-0.27	0.82	-9.08	-2.92	-3.62	-37.48	-59.43	3.88	0.45	-5.23	-14.11	-0.45	-5.00	-0.76	-2.11	-27.72	-40.04
Minimum	-25.36	-26.21	-22.70	-30.03	-24.93	-25.91	-29.79	-34.74	-86.44	-86.91	-33.74	-41.80	-34.35	-37.77	-23.86	-26.26	-20.97	-21.07	-76.52	-93.54
Maximum	29.34	16.05	47.88	37.33	31.67	29.55	48.77	41.88	479.83	454.17	92.04	90.24	57.89	54.46	33.08	29.84	27.52	17.85	36.86	34.89
Average	4.81	-0.64	11.94	2.65	0.04	-5.76	-0.29	-6.66	101.86	54.02	16.65	4.66	10.52	1.85	-0.74	-5.56	4.78	-0.03	-2.07	-8.37

Table B5. Hybrid entries in 2018 F_2 experiment with their respective mid-parent (MPH) and high-parent heterosis (HPH) estimates calculated from single locations, locations groups together according to GGE biplot analysis and all locations combined.

	BD		LN		MCG		PRO		LNMCG		Combined		F1	
Hybrid entries	**MPH**	**HPH**	**MPH**	**HPH**	**MPH**	**HPH**	**MPH**	**HPH**	**MPH**	**HPH**	**MPH**	**HPH**	**MPH**	**HPH**
NXB15-6162	5.21	-0.91	-1.13	-6.37	-7.35	-9.57	7.03	-2.09	-1.79	-3.32	-0.35	-2.16	-6.08	-8.86
NXB15-6163	7.97	-3.79	-1.11	-2.91	-23.76	-29.44	-111.35	-130.61	-11.27	-13.02	-8.86	-11.04	-3.39	-6.29
NXB15-6502	-11.92	-22.80	-4.09	-4.92	7.35	0.88	-79.97	-165.93	0.47	-2.27	0.57	-1.23	21.81	17.95
NXB15-6534	1.75	-7.83	-1.94	-13.49	4.28	-6.21	-14.72	-81.51	3.98	-6.14	-1.81	-7.19	0.34	-8.40
NXB15-6563	1.08	-8.57	4.90	-7.40	18.36	16.78	-7.18	-42.12	6.85	-0.78	3.65	-0.12	-16.06	-26.66
NXB15-6567	5.09	-3.55	13.29	9.29	9.52	8.07	16.22	12.04	13.32	12.57	9.17	5.90	-4.00	-14.24
NXB15-6583	18.74	10.82	5.48	-5.23	-16.29	-29.03	-38.94	-119.83	1.13	-9.29	3.17	-1.94	0.41	-8.32
NXB15-6699	10.24	9.74	1.23	-5.36	3.75	-2.25	-14.47	-36.94	3.62	2.64	2.64	1.46	20.71	16.37
NXB15-6754	-5.18	-16.51	2.75	-0.65	13.96	13.11	-94.25	-100.27	6.77	5.01	1.62	0.16	-5.61	-13.94
NXB15-6759	-9.73	-13.95	-0.74	-3.26	10.21	2.87	36.89	35.46	6.05	4.49	4.29	3.02	0.38	-2.83
NXB15-6822	-12.17	-22.52	-18.13	-22.65	15.49	14.65	-31.37	-52.34	-3.57	-8.65	-5.18	-7.19	9.22	7.98
NXB15-6931	9.17	0.90	2.14	-2.38	18.31	17.00	-228.71	-245.09	10.66	9.89	2.30	-1.22	2.14	-7.50
NXB15-6950	8.26	5.76	6.49	0.52	0.21	-1.84	4.90	-32.86	7.87	1.91	5.65	5.48	2.80	-9.84
NXB15-6955	-12.01	-22.16	6.59	1.04	18.62	16.66	-3.75	-23.80	13.67	7.68	6.79	6.68	20.64	15.86
NXB15-7126	-3.29	-13.37	-13.82	-28.54	1.79	-0.11	15.56	-11.97	-5.21	-13.83	-1.80	-5.79	4.13	-4.63
NXB15-7175	-10.64	-17.47	-1.87	-4.37	8.94	6.01	26.59	23.43	4.10	3.95	2.34	2.09	11.44	5.15
NXB15-7186	-8.70	-11.38	5.31	-0.32	17.03	13.42	-2.63	-27.36	9.51	4.07	5.89	0.10	18.58	15.93
NXB15-7222	-4.37	-11.61	-3.79	-7.19	-16.82	-27.03	-31.67	-32.86	-7.12	-9.32	-6.17	-8.92	13.28	13.24
NXB15-7230	7.24	0.78	-7.16	-14.97	-27.41	-43.60	-28.59	-48.34	-16.26	-27.69	-9.90	-16.57	-10.40	-14.70
NXB15-7295	-7.81	-14.69	-0.72	-2.90	-8.20	-21.23	59.27	51.88	-3.68	-8.68	-0.42	-4.35	7.48	4.70
NXB15-7298	3.40	2.54	6.38	-0.47	-9.39	-19.72	18.65	-23.99	2.83	-5.01	1.83	-6.27	1.52	-3.59
NXB15-7310	0.02	-3.34	-1.77	-3.17	8.64	3.97	-12.43	-48.88	3.71	1.73	2.05	-0.02	23.86	22.39
NXB15-7426	-2.62	-12.11	-12.38	-16.01	-8.95	-13.07	-44.87	-46.52	-11.74	-12.00	-9.34	-10.43	-12.91	-17.36
Min	-12.17	-22.80	-18.13	-28.54	-27.41	-43.60	-228.71	-245.09	-16.26	-27.69	-9.90	-16.57	-16.06	-26.66
Max	18.74	10.82	13.29	9.29	18.62	17.00	59.27	51.88	13.67	12.57	9.17	6.68	23.86	22.39
Average	-0.45	-7.65	-0.61	-6.16	1.66	-3.90	-24.34	-50.02	1.47	-2.87	0.35	-2.59	4.36	-1.20

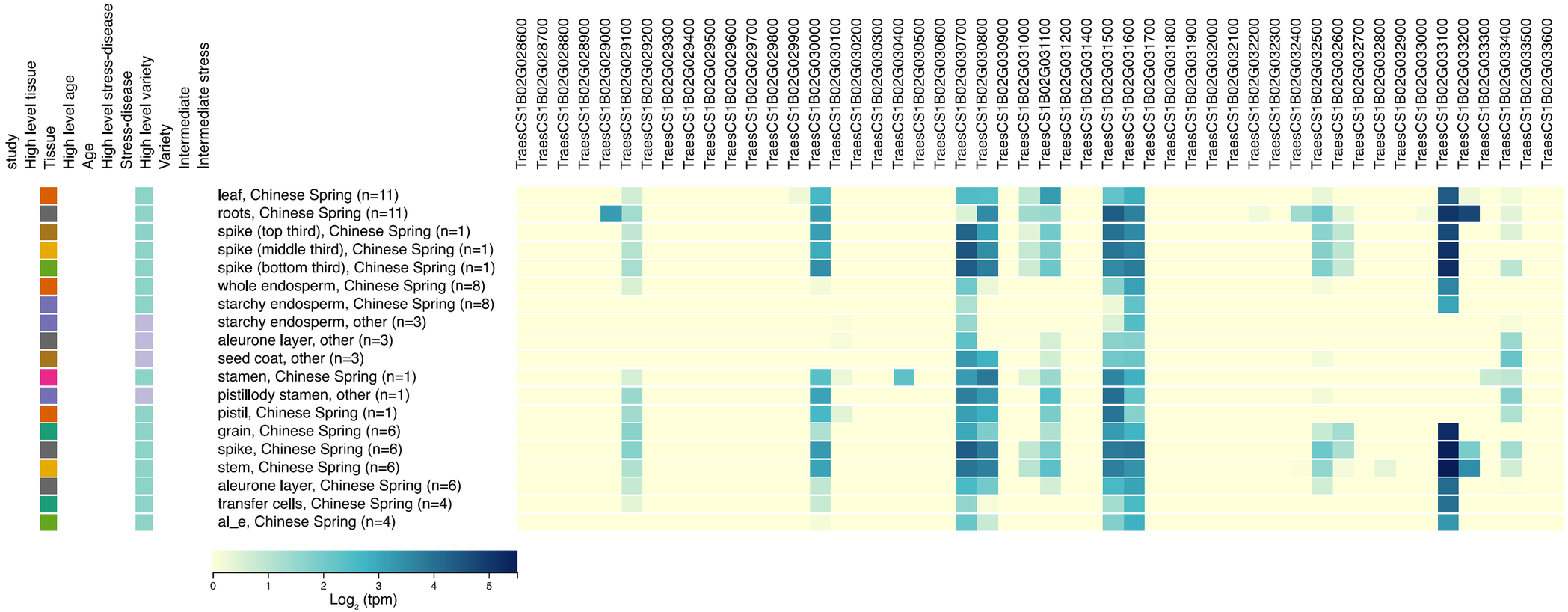

Figure C1. Heatmap of RNA seq expression data extracted for genes within QTL in 1B in Chinese Spring. The columns represent genes whereas rows represent tissue type from which RNA was extracted and sequenced. The color ranges from yellow to blue and represent RNA reads in millions.

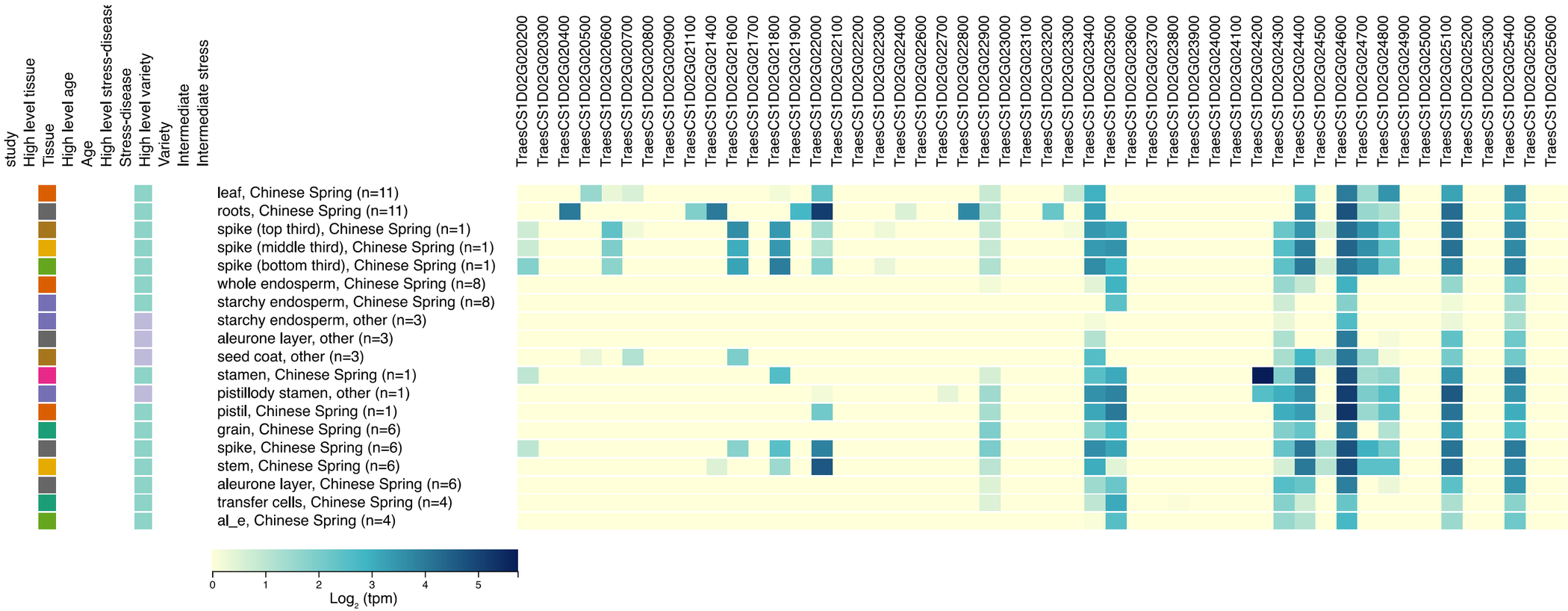

Figure C2. Heatmap of RNA seq expression data extracted for genes within QTL 1B in Chinese Spring. The columns represent genes whereas rows represent tissue type from which RNA was extracted and sequenced. The color ranges from yellow to blue and represent RNA reads in millions.

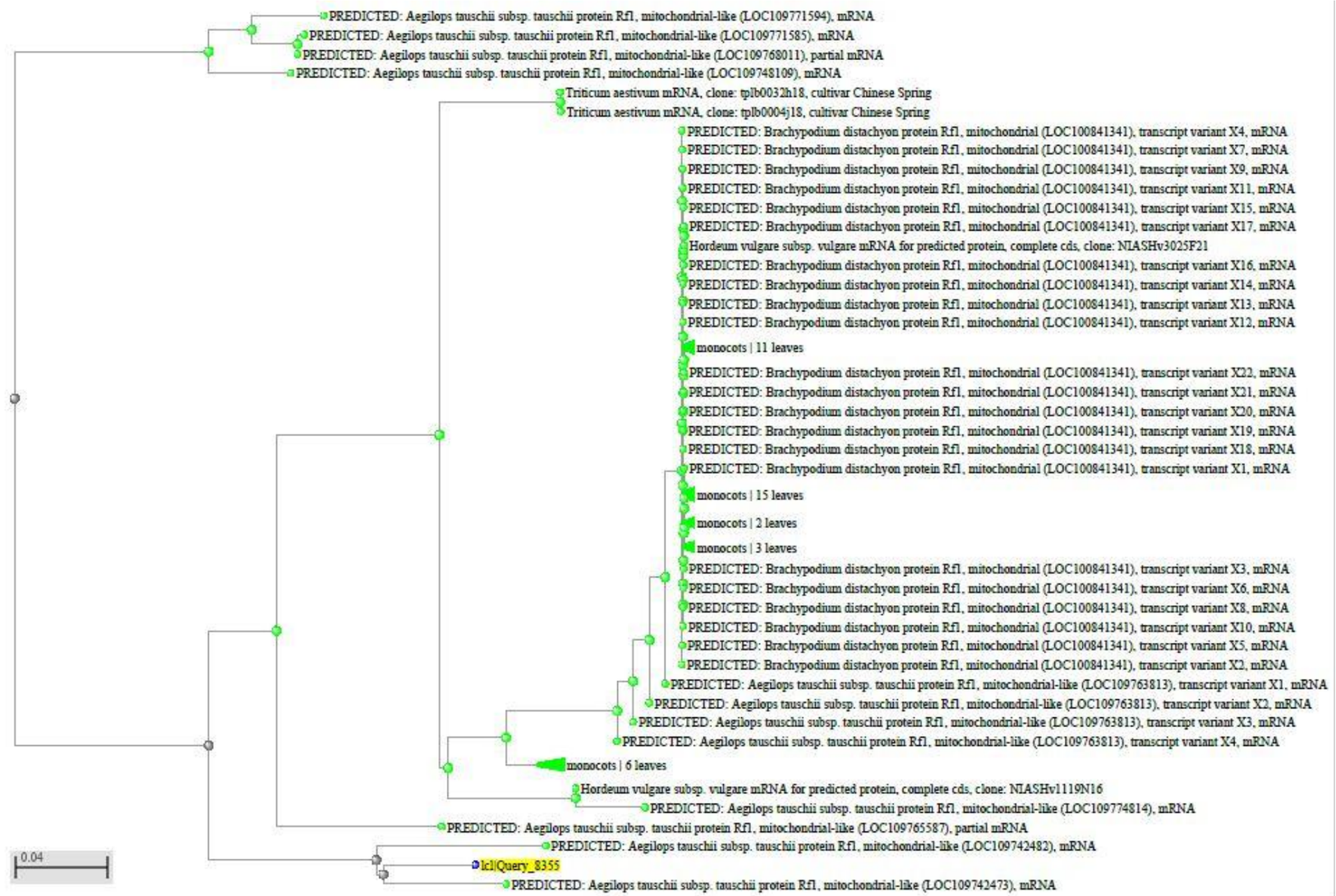

Figure C3. Neighbor joining tree of BLAST search results for pentatricopeptide repeat (PPR) gene TraesCS1B02G038200 identified within the QTL confidence interval in 1B. The branch highlighted in yellow is the sequence of TraesCS1B02G038200.

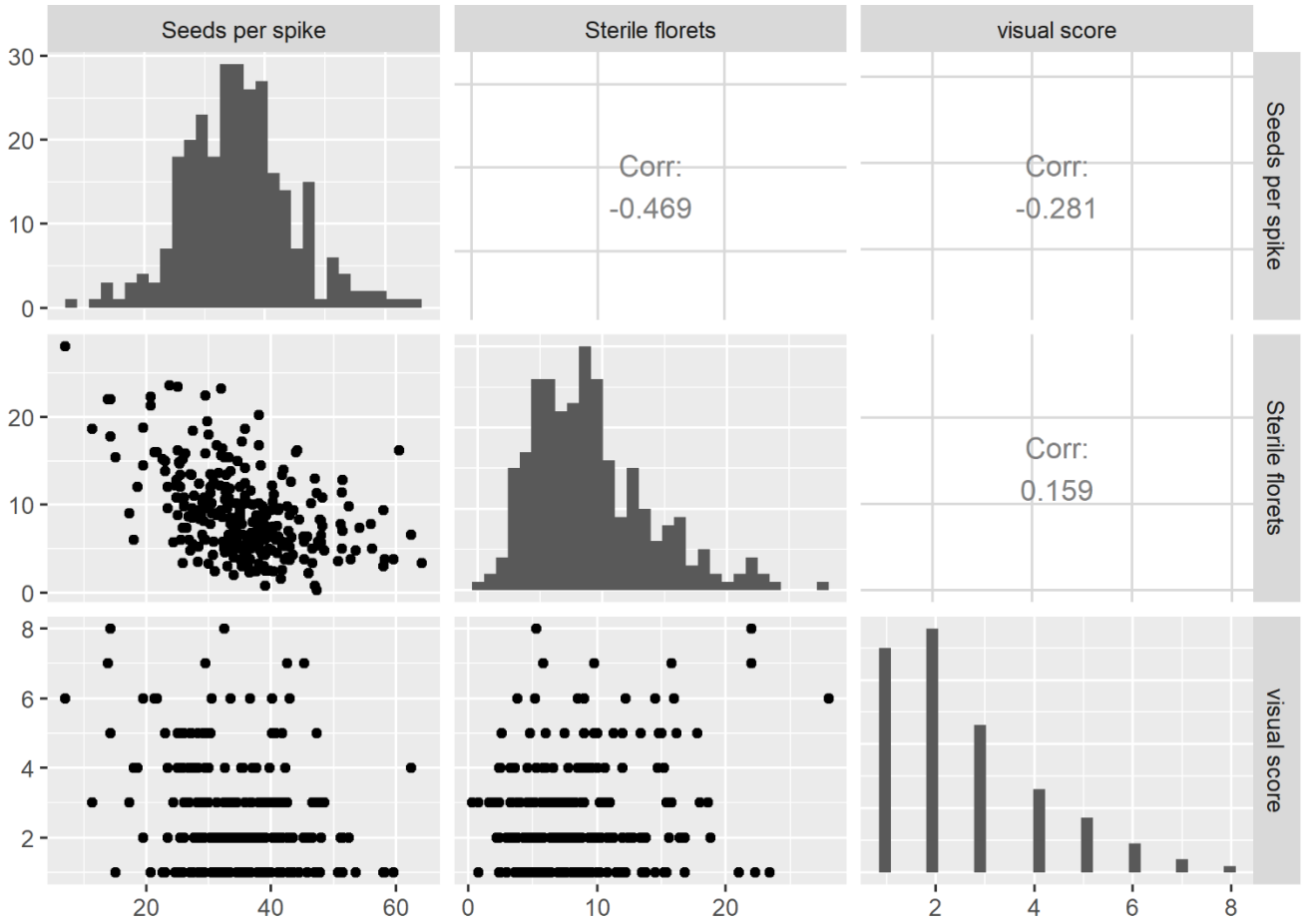

Figure C4. Distribution and correlation coefficients of RILs phenotyping in El Batan in 2018. The traits considered are seeds per spike, sterile florets per spike and sterility visual score.

Table C1. Phenotypic data that came from six experiments conducted at El Batan, Obregon and Toluca, Mexico in 2018-19 for assessing fertility restoration in a recombinant inbred line (RILs) mapping population derived from Cargill115 x C80 cross at International Maize and Wheat Improvement Center (CIMMYT), Mexico. Phenotypic data were collected from an experiment where RILs were evaluated and from four experiments in which test cross progenies from crosses between RILs and a cytoplasmic male sterile line were evaluated for fertility restoration. Phenotypic data was collected on average seeds per spike, average sterile florets per spike and a visual score (1-10, 1 = fertile, 10 = sterile). Data for combined analysis for visual score came from five test cross evaluation experiments whereas combined analysis for seed per spike came from El Batan in 2018 and Obregon in 2019.

Experiment	RILs evaluation El Batan 2018			Test cross evaluation El Batan 2018			Test cross evaluation El Batan 2019		Test cross evaluation Obregon 2019		Test cross evaluation Toluca 2019		Combined Analysis	
RIL entry number	Sterile florets per spike	Seed per spike	Sterility visual score	Sterile florets per spike	Seed per spike	Sterility visual score	Sterility visual score	Seed per spike	Sterility visual score	Seed per spike	Sterility visual score	Seed per spike	Sterility visual score	Seed per spike
1	12.0	32.8	1	15.4	47.2	2	2.9	65.7	2	62.1	1.9	64.5	2.3	59.87
2	8.5	38.3	1	36.8	5.0	8	8.0	7.9	-	-	8.4	0.8	8.2	43.94
3	6.4	41.2	1	-	-	-	2.0	64.6	1	71.1	1.4	60.8	1.5	42.83
4	4.6	33.8	3	26.5	4.5	9	-	17.0	8	19.6	-	-	8.5	44.54
5	9.8	38.6	2	7.9	46.4	2	-	-	1	74.2	-	-	1.5	47.83
6	16.2	44.2	-	12.4	43.3	2	9.0	15.3	-	44.5	8.5	0.1	6.5	54.58
7	9.4	35.0	2	32.8	4.3	9	8.8	3.2	-	53.3	8.8	3.6	8.9	6.58
8	9.4	58.0	1	-	-	-	8.9	5.6	7	25.7	9.9	0.7	8.7	43.39
9	9.4	41.2	2	-	-	4	8.9	1.4	9	17.8	-	-	7.3	23.85
10	12.0	35.0	-	18.2	41.9	2	4.0	45.7	2	58.7	3.5	29.5	2.9	6.19
11	6.4	38.8	2	3.1	59.6	2	3.3	33.5	1	76.1	4.5	35.4	2.7	8.97
12	5.2	28.4	1	24.5	7.6	10	4.8	13.5	5	40.2	7.5	10.9	6.8	59.43
13	20.2	38.0	-	-	-	-	1.9	72.4	-	73.5	-	-	2.0	51.14
14	3.8	45.3	2	12.0	33.8	6	2.0	47.1	1	59.6	2.5	68.1	2.9	8.63
15	2.0	34.0	3	29.3	7.8	9	7.8	9.1	3	58.3	8.5	4.1	7.1	32.53
16	18.6	11.3	3	26.4	2.7	9	2.0	41.4	2	60.7	2.3	37.6	3.8	59.41
17	23.6	23.7	-	32.8	5.1	4	5.0	0.0	-	-	8.8	0.5	5.9	8.99
18	-	-	-	18.6	32.4	2	1.8	53.0	2	71.8	3.5	53.8	2.3	24.68
19	6.8	36.8	2	-	-	-	9.8	0.8	-	42.7	9.7	0.1	9.8	47.05
20	3.0	33.0	1	6.1	35.3	7	1.0	46.5	1	69.9	1.8	50.8	2.7	26.43

Table C1. Continued

Experiment	RILs evaluation El Batan 2018			Test cross evaluation El Batan 2018			Test cross evaluation El Batan 2019		Test cross evaluation Obregon 2019		Test cross evaluation Toluca 2019		Combined Analysis	
RIL entry number	Sterile florets per spike	Seed per spike	Sterility visual score	Sterile florets per spike	Seed per spike	Sterility visual score	Sterility visual score	Seed per spike	Sterility visual score	Seed per spike	Sterility visual score	Seed per spike	Sterility visual score	Seed per spike
21	7.6	39.0	1	-	-	-	2.3	47.9	-	-	3.3	50.3	2.8	5.20
22	9.6	27.3	1	8.2	54.7	2	1.9	62.1	1	49.1	-	-	1.7	75.10
23	13.6	31.6	1	11.3	36.3	3	4.0	29.2	2	46.2	5.3	4.0	3.6	38.09
24	5.6	36.0	1	34.8	0.1	10	8.0	5.3	5	41.2	8.9	0.6	8.0	18.04
25	10.6	29.2	3	17.6	34.9	4	8.3	5.6	2	38.6	8.8	2.0	5.8	55.01
26	12.2	24.6	1	8.9	33.9	3	1.5	55.3	2	47.4	2.3	62.2	2.2	-0.75
27	3.4	25.8	2	-	-	-	10.0	0.0	9	1.8	10.0	0.0	9.7	57.44
28	15.8	26.2	3	20.7	17.2	8	2.0	55.8	2	79.1	2.5	56.5	3.6	56.51
29	5.0	46.6	2	34.8	0.4	9	9.5	2.4	7	36.1	10.0	0.5	8.9	21.37
30	10.2	30.5	1	6.9	32.7	4	1.8	52.8	2	52.4	2.8	52.7	2.7	50.55
31	5.0	32.5	3	-	-	-	1.8	51.9	-	-	3.4	60.1	2.7	50.23
32	4.8	53.6	1	-	-	-	1.5	56.4	1	66.9	2.3	68.4	1.6	48.51
33	15.4	15.0	1	27.2	4.2	8	2.5	47.5	-	35.1	3.5	35.8	4.7	48.11
34	7.6	48.2	-	12.8	29.5	6	2.0	60.2	1	71.5	2.0	55.7	2.8	50.49
35	11.8	33.4	1	-	-	8	6.0	30.2	-	-	7.3	12.5	7.1	66.90
36	8.8	35.0	-	6.7	42.3	2	9.5	0.4	-	-	8.5	2.5	6.7	36.43
37	3.8	59.6	1	26.7	22.7	5	4.5	46.5	4	53	3.9	40.0	4.4	8.78
38	10.0	26.0	2	31.3	0.3	9	9.0	3.0	6	39.4	9.4	1.4	8.4	21.85
39	12.8	51.5	2	20.7	22.2	7	-	-	7	50.3	-	-	7.0	NA
40	12.5	35.8	2	-	-	6	4.0	46.4	3	59.4	5.0	30.4	4.5	-1.96
41	-	-	-	23.8	30.8	-	-	-	-	-	-	-	-	15.40
42	13.4	41.8	-	-	-	-	9.5	0.2	-	-	9.2	0.1	9.4	41.04
43	13.4	25.4	2	15.2	19.8	7	2.5	41.7	1	66.7	2.8	42.0	3.3	38.74
44	14.5	19.5	6	3.9	39.1	4	3.5	55.6	-	57.3	2.8	50.8	3.4	49.85
45	9.2	28.0	4	32.6	0.0	9	9.0	0.7	-	45.2	9.3	0.0	9.1	0.56
46	6.0	39.0	1	18.9	13.4	8	5.4	57.0	5	45.2	-	-	6.2	52.14
47	13.5	30.3	-	14.7	38.3	3	4.9	22.2	-	-	8.9	4.6	5.7	32.46

Table C1. Continued

Experiment	RILs evaluation El Batan 2018			Test cross evaluation El Batan 2018			Test cross evaluation El Batan 2019		Test cross evaluation Obregon 2019		Test cross evaluation Toluca 2019		Combined Analysis	
RIL entry number	Sterile florets per spike	Seed per spike	Sterility visual score	Sterile florets per spike	Seed per spike	Sterility visual score	Sterility visual score	Seed per spike	Sterility visual score	Seed per spike	Sterility visual score	Seed per spike	Sterility visual score	Seed per spike
48	10.8	35.8	2	23.6	9.0	8	7.0	7.3	-	0.3	7.8	8.5	7.6	5.25
49	6.4	40.4	2	33.2	0.5	9	7.8	6.3	-	59	8.8	1.5	8.5	70.99
50	14.0	42.0	-	19.3	21.8	8	8.0	3.9	-	-	9.0	0.9	8.3	3.99
51	8.0	34.5	2	-	-	-	2.0	44.5	2	69.2	1.0	56.9	1.7	8.64
52	5.4	47.7	1	-	-	-	7.5	12.4	-	-	8.8	1.1	8.2	62.86
53	12.0	25.4	2	32.9	12.1	3	8.9	10.0	-	-	-	-	6.0	57.48
54	6.4	45.8	1	33.1	7.0	8	9.0	3.1	-	56.5	9.2	0.6	8.8	28.98
55	7.0	51.5	1	29.8	5.6	8	7.9	36.2	9	37	9.9	0.0	8.8	47.51
56	11.2	40.5	-	-	-	-	9.0	0.0	-	-	8.5	0.2	8.8	45.64
57	9.0	28.6	1	15.6	30.1	4	5.5	26.4	-	-	6.3	12.5	5.3	19.83
58	12.0	18.6	4	9.9	46.8	4	9.5	0.0	10	7.2	9.3	0.1	8.2	22.88
59	9.4	32.0	1	-	-	-	9.0	1.5	-	36.6	9.5	0.6	9.3	53.03
60	10.8	33.0	1	10.5	39.8	3	1.4	60.7	2	54.2	3.4	62.8	2.5	26.08
61	10.8	33.2	2	-	-	-	1.8	55.2	4	16.7	2.5	59.9	2.8	18.28
62	8.6	32.2	1	27.1	18.1	7	4.8	37.0	3	37.9	6.0	28.5	5.2	63.03
63	-	-	-	22.9	19.1	4	-	-	-	-	-	-	4.0	45.68
64	-	-	2	-	-	-	3.8	39.7	-	86.5	6.0	30.5	4.9	46.16
65	4.4	38.5	1	22.9	14.8	7	5.4	15.5	6	41.5	7.4	3.7	6.5	6.51
66	8.6	35.0	2	9.3	47.7	3	2.5	52.0	-	68.5	3.4	40.7	3.0	13.29
67	10.8	48.2	-	-	-	-	2.5	59.0	1	68.2	3.8	56.0	2.4	19.00
68	9.4	35.0	1	-	-	-	7.5	14.5	4	59.4	7.2	15.7	6.3	35.59
69	11.0	27.7	2	3.0	45.8	3	1.4	66.7	1	53.1	-	-	1.8	60.40
70	13.8	23.0	1	31.8	0.1	9	7.4	9.3	5	46.9	-	-	7.2	4.00
71	18.6	35.8	-	-	-	-	1.8	58.7	2	68.2	3.3	37.0	2.4	55.46
72	0.8	47.0	1	11.6	33.8	3	3.0	46.5	2	79.6	3.5	40.6	2.9	25.98

Table C1. Continued

Experiment	RILs evaluation El Batan 2018			Test cross evaluation El Batan 2018			Test cross evaluation El Batan 2019		Test cross evaluation Obregon 2019		Test cross evaluation Toluca 2019		Combined Analysis	
RIL entry number	Sterile florets per spike	Seed per spike	Sterility visual score	Sterile florets per spike	Seed per spike	Sterility visual score	Sterility visual score	Seed per spike	Sterility visual score	Seed per spike	Sterility visual score	Seed per spike	Sterility visual score	Seed per spike
73	16.2	60.5	-	-	-	-	1.5	68.3	-	75.5	2.3	59.9	1.9	5.40
74	9.4	43.6	1	30.3	0.9	6	9.0	1.1	8	21.2	9.5	0.3	8.1	21.53
75	15.0	34.6	-	37.6	1.7	6	1.8	61.1	1	67.4	1.0	59.8	2.5	17.50
76	7.8	30.3	2	5.0	47.8	2	1.8	51.8	1	69	1.8	57.6	1.7	45.44
77	3.8	52.8	-	-	-	-	5.9	19.0	-	-	8.5	2.9	7.3	67.33
78	5.2	35.8	3	6.1	41.4	5	3.0	46.1	-	75	4.3	70.5	4.1	57.31
79	8.8	34.8	2	10.4	40.0	4	2.0	45.0	1	63	2.5	49.8	2.4	6.91
80	17.8	14.2	5	27.1	11.6	5	10.0	1.0	-	24.5	9.8	0.2	8.3	17.48
81	9.5	40.8	2	19.1	17.4	8	9.5	0.6	7	38.1	9.8	0.1	8.6	18.15
82	14.5	38.3	1	-	-	-	1.8	62.1	2	61.6	1.8	66.4	1.9	18.57
83	8.8	31.3	3	6.5	37.4	5	1.9	49.8	1	45.5	-	-	2.7	28.76
84	4.6	32.6	4	9.0	31.8	6	1.4	52.0	1	43.7	-	-	2.8	30.98
85	13.4	27.2	5	-	-	-	5.9	21.5	-	50.6	5.4	46.2	5.8	2.45
86	15.2	22.6	1	24.6	11.1	6	1.0	54.1	-	-	1.5	74.1	2.8	17.86
87	18.4	27.4	-	-	-	-	2.0	51.5	-	-	2.5	47.7	2.3	57.25
88	14.8	25.2	5	27.1	8.1	8	9.0	1.3	9	12.4	9.5	0.3	8.9	10.56
89	5.0	34.6	2	25.0	2.8	9	8.0	11.0	7	38.7	9.3	2.9	8.3	28.06
90	9.0	28.2	3	6.3	33.8	4	1.9	48.4	2	29	3.9	51.0	3.0	52.75
91	11.6	36.7	1	7.2	59.0	2	2.3	54.3	2	56.7	1.8	58.1	2.0	9.90
92	10.3	40.3	2	7.3	37.5	4	2.5	43.7	2	68.9	3.3	46.0	3.0	12.79
93	7.2	37.8	1	11.7	24.7	7	2.3	60.1	1	69.8	3.3	67.1	3.4	19.39
94	10.2	34.4	3	36.6	1.0	8	8.9	1.4	9	0	-	-	8.7	17.33
95	8.3	44.5	1	34.3	12.7	6	8.3	10.5	3	52.6	8.0	4.1	6.3	40.35
96	6.2	36.8	3	-	-	-	4.5	29.5	3	55	6.5	19.6	4.7	9.23
97	6.0	29.3	1	-	-	-	2.3	53.2	1	45.7	1.5	49.9	1.6	36.50
98	17.2	35.3	-	32.5	14.4	3	3.0	36.6	1	46.2	4.5	11.5	2.9	51.72
99	15.4	33.2	1	10.3	30.2	3	2.8	40.3	1	41.6	3.0	35.1	2.5	10.11

Table C1. Continued

Experiment	RILs evaluation El Batan 2018			Test cross evaluation El Batan 2018			Test cross evaluation El Batan 2019		Test cross evaluation Obregon 2019		Test cross evaluation Toluca 2019		Combined Analysis	
RIL entry number	Sterile florets per spike	Seed per spike	Sterility visual score	Sterile florets per spike	Seed per spike	Sterility visual score	Sterility visual score	Seed per spike	Sterility visual score	Seed per spike	Sterility visual score	Seed per spike	Sterility visual score	Seed per spike
100	6.6	33.8	3	14.9	29.4	7	2.0	46.2	1	46.1	2.8	49.6	3.2	10.28
101	5.0	36.0	1	3.3	40.5	6	2.8	47.2	4	35	2.0	55.5	3.7	12.01
102	13.0	47.0	-	9.4	33.5	2	2.0	46.3	-	-	2.3	48.6	2.1	59.91
103	5.8	41.8	1	13.6	32.3	2	2.4	66.4	1	45.4	1.9	74.2	1.9	45.45
104	8.7	26.7	4	-	-	-	9.4	5.6	8	20.5	9.4	1.2	9.0	20.41
105	5.8	30.8	2	-	-	-	3.3	38.5	1	67.4	3.3	31.9	2.5	28.38
106	8.5	27.3	4	22.0	25.3	5	4.3	21.4	-	-	5.9	9.7	5.1	3.79
107	7.4	54.2	-	-	-	-	8.0	4.3	-	-	8.8	0.5	8.4	6.15
108	22.3	20.7	1	34.0	0.4	9	10.0	8.9	6	25.4	9.3	1.2	8.6	57.24
109	10.2	38.0	-	6.5	57.6	-	2.0	54.5	-	-	1.7	51.0	1.9	29.14
110	10.0	29.6	5	32.3	5.1	9	9.0	2.1	6	27.1	10.0	0.2	8.5	7.01
111	16.0	44.0	-	11.4	54.8	-	6.0	7.6	-	-	5.5	20.1	5.8	61.38
112	8.0	34.5	1	7.1	57.4	2	2.0	46.0	5	-	1.5	59.7	2.6	9.61
113	5.3	35.5	4	29.5	0.4	9	8.3	2.8	5	32.4	9.3	0.4	7.9	50.63
114	7.2	34.0	2	3.0	49.0	3	7.3	5.8	5	39.9	7.7	4.0	5.8	44.67
115	5.8	47.5	1	29.2	3.3	8	1.8	46.3	1	72.6	1.8	66.0	3.2	61.59
116	6.6	33.2	3	-	-	-	8.0	19.7	5	61.1	7.8	6.0	6.9	50.45
117	22.0	13.8	7	36.0	0.0	9	9.5	0.4	-	-	8.8	0.1	9.1	20.27
118	13.4	32.8	-	5.4	68.6	-	2.9	71.0	-	-	-	-	3.0	58.10
120	14.2	35.8	-	10.2	50.5	-	5.0	51.6	2	72.1	3.5	45.9	3.5	18.99
121	4.8	40.2	5	33.3	0.0	7	9.4	1.5	-	0.8	-	-	8.3	18.45
122	4.8	38.2	2	14.4	34.6	6	2.5	62.5	1	63	2.8	69.7	3.1	12.54
123	10.2	36.2	1	-	-	-	2.0	59.7	2	63.1	3.3	54.3	2.4	49.08
124	-	-	-	25.4	13.8	5	-	-	-	-	-	-	5.0	52.91
119	21.3	20.7	-	-	-	-	4.0	43.3	-	-	5.0	25.3	4.5	69.84
125	7.6	34.6	2	28.4	13.1	7	1.4	56.0	1	66.5	2.4	66.6	3.0	50.65
126	2.4	37.6	2	33.9	0.9	8	1.8	61.8	1	60.2	1.9	78.0	3.2	6.00

Table C1. Continued

Experiment	RILs evaluation El Batan 2018			Test cross evaluation El Batan 2018			Test cross evaluation El Batan 2019		Test cross evaluation Obregon 2019		Test cross evaluation Toluca 2019		Combined Analysis	
RIL entry number	Sterile florets per spike	Seed per spike	Sterility visual score	Sterile florets per spike	Seed per spike	Sterility visual score	Sterility visual score	Seed per spike	Sterility visual score	Seed per spike	Sterility visual score	Seed per spike	Sterility visual score	Seed per spike
127	12.4	28.5	2	-	-	-	1.5	45.3	1	68	2.3	39.8	1.6	16.85
128	19.5	29.8	-	-	-	-	3.5	33.1	-	-	5.3	55.6	4.4	5.74
129	4.3	39.0	1	11.3	34.3	6	1.5	49.5	1	68	1.0	50.2	2.4	7.15
130	6.6	35.4	2	-	-	-	3.8	34.9	1	64.8	5.0	17.2	3.3	31.81
131	2.4	37.0	4	27.9	2.9	9	7.8	3.4	-	-	10.0	4.9	8.9	49.25
132	12.2	31.8	1	33.2	13.1	6	8.5	8.0	3	59.8	9.3	6.5	6.7	5.11
133	-	-	-	-	-	-	-	-	-	-	-	-	-	15.31
134	18.0	30.0	3	-	-	-	9.4	1.4	9	0.2	8.9	0.1	9.2	26.46
135	9.0	28.3	5	29.3	2.9	9	8.9	17.3	-	-	-	-	9.0	53.79
136	5.4	41.8	2	-	-	-	4.0	36.5	1	69.3	5.0	24.9	3.3	26.93
137	8.8	28.8	1	17.0	26.1	5	3.5	39.4	1	58.7	3.5	30.8	3.3	37.56
138	15.6	32.0	2	7.0	56.7	2	4.5	39.3	2	58.7	4.5	44.7	3.3	56.58
139	7.0	42.8	1	34.0	0.0	9	10.0	0.0	10	2.1	9.7	0.1	9.7	15.80
140	4.8	27.0	5	20.2	18.8	3	3.0	32.5	-	-	3.8	31.0	3.3	18.53
141	28.0	7.0	6	31.0	0.4	8	9.3	0.1	-	-	9.5	0.1	8.9	46.31
142	6.0	34.5	3	-	-	-	2.9	67.5	-	-	1.4	66.9	2.3	21.40
143	18.8	19.5	2	-	-	-	9.2	0.2	-	-	9.8	0.2	9.6	20.05
147	22.4	29.5	-	32.1	21.5	-	4.5	30.1	-	-	4.5	20.2	4.5	57.80
148	6.4	39.4	3	12.2	40.4	3	3.0	41.6	-	-	3.9	45.4	3.3	44.86
149	9.6	33.0	2	10.7	43.1	2	2.8	42.3	2	61.2	3.5	36.0	2.6	29.33
150	6.4	39.6	1	27.8	6.4	6	6.3	22.4	4	50.4	7.3	12.3	5.9	52.96
151	8.2	29.2	2	13.3	34.0	3	3.3	50.3	-	-	3.5	59.7	3.3	7.56
152	7.8	51.2	2	21.7	23.2	5	7.0	31.2	-	1.5	4.8	48.4	5.6	5.11
153	16.4	32.2	2	34.9	7.3	4	10.0	0.0	1	65.8	9.2	0.0	6.1	31.83
144	7.4	26.4	3	-	-	-	6.8	6.6	-	-	7.5	3.1	7.2	23.13
145	6.6	29.0	3	-	-	-	2.0	47.7	-	-	2.5	70.5	2.3	56.05
146	13.5	27.0	1	3.5	52.8	2	2.0	48.5	1	62.4	1.0	66.2	1.5	28.91

Table C1. Continued

Experiment	RILs evaluation El Batan 2018			Test cross evaluation El Batan 2018			Test cross evaluation El Batan 2019		Test cross evaluation Obregon 2019		Test cross evaluation Toluca 2019		Combined Analysis	
RIL entry number	Sterile florets per spike	Seed per spike	Sterility visual score	Sterile florets per spike	Seed per spike	Sterility visual score	Sterility visual score	Seed per spike	Sterility visual score	Seed per spike	Sterility visual score	Seed per spike	Sterility visual score	Seed per spike
154	8.4	28.6	2	6.7	42.3	2	2.0	52.8	-	-	1.3	78.9	1.8	45.48
155	3.3	30.0	4	8.3	42.4	3	2.8	45.4	-	-	2.3	34.1	2.7	53.33
156	9.8	26.0	5	8.2	31.7	5	3.0	46.6	-	-	4.3	45.1	4.1	55.02
157	12.6	30.8	2	31.9	1.6	9	9.9	2.8	-	-	8.9	0.0	9.3	11.80
158	2.3	36.5	2	-	-	-	9.0	16.7	-	-	8.7	2.3	8.9	3.84
159	3.6	35.2	4	26.4	5.6	8	7.4	21.8	-	-	-	-	7.8	39.20
160	9.4	40.0	1	6.2	50.8	2	2.0	59.3	1	63.3	1.5	68.2	1.6	58.51
161	11.3	47.3	5	35.8	0.0	-	10.0	0.0	6	16	9.0	0.0	8.3	47.71
162	3.3	36.3	2	8.6	45.3	2	1.8	46.5	-	-	1.3	59.5	1.7	9.31
163	6.3	38.8	1	34.8	1.9	8	5.4	39.5	-	-	6.9	21.4	6.8	26.74
164	3.0	38.8	2	30.9	1.0	8	9.3	0.0	-	-	9.3	0.1	8.9	59.76
165	8.3	38.7	3	14.6	19.9	7	5.8	20.4	-	-	7.3	9.1	6.7	44.76
166	16.8	38.0	1	33.9	6.9	7	6.9	17.5	-	-	-	-	7.0	28.21
167	8.3	47.8	1	18.7	31.0	3	2.3	42.6	1	45.6	1.8	62.6	2.0	19.07
168	3.4	64.2	-	9.3	42.1	2	2.5	66.3	-	-	2.0	78.5	2.2	20.29
174	9.8	29.2	5	11.6	33.4	2	4.8	25.3	-	-	4.0	19.1	3.6	17.53
175	8.3	35.0	2	36.1	3.1	8	9.8	0.1	-	6.6	9.7	0.0	9.2	36.51
176	10.0	37.0	4	-	-	-	7.3	27.5	7	28.1	7.0	5.5	7.1	12.68
177	10.8	29.3	2	4.1	36.9	2	5.0	67.0	-	-	-	-	3.5	20.10
178	7.8	34.0	3	31.1	9.9	8	9.8	5.6	-	-	8.5	1.1	8.8	6.41
179	10.8	26.0	3	21.3	30.4	7	7.5	26.9	-	-	6.8	11.8	7.1	49.70
180	9.6	41.3	-	30.0	12.7	6	8.0	1.8	-	-	8.8	0.0	7.6	5.19
181	2.4	31.0	1	25.7	23.3	7	8.3	2.1	7	24.2	8.8	1.5	7.8	53.86
182	3.0	58.0	-	-	-	-	9.5	6.1	-	57.8	8.5	1.8	9.0	17.70
169	6.4	45.2	2	7.1	55.6	2	2.0	60.8	1	53.1	2.3	59.8	1.8	20.80
170	8.8	25.0	4	17.0	33.7	6	9.5	0.0	-	-	9.8	3.6	8.4	5.91
171	4.3	30.7	2	28.4	6.1	7	5.4	20.6	-	-	6.9	12.6	6.5	22.79

Table C1. Continued

Experiment	RILs evaluation El Batan 2018			Test cross evaluation El Batan 2018			Test cross evaluation El Batan 2019		Test cross evaluation Obregon 2019		Test cross evaluation Toluca 2019		Combined Analysis	
RIL entry number	Sterile florets per spike	Seed per spike	Sterility visual score	Sterile florets per spike	Seed per spike	Sterility visual score	Sterility visual score	Seed per spike	Sterility visual score	Seed per spike	Sterility visual score	Seed per spike	Sterility visual score	Seed per spike
172	12.6	43.2	-	29.0	11.0	6	-	-	-	-	-	-	6.0	0.30
173	11.3	30.3	2	3.8	40.3	2	6.3	22.8	3	47.9	7.8	4.1	4.8	17.31
183	4.3	43.5	2	21.7	24.7	6	7.3	10.5	-	-	9.0	1.6	7.4	NA
184	7.8	56.0	-	8.6	49.1	2	8.0	6.5	-	54.8	8.3	51.0	6.1	11.45
185	5.0	56.2	-	30.4	4.4	-	9.5	6.2	-	-	8.8	1.9	9.2	14.18
186	7.0	40.2	3	24.0	22.3	5	3.5	38.8	-	62.4	4.8	22.5	4.4	62.76
187	1.6	41.6	3	5.1	43.1	3	2.4	47.8	1	68.8	-	-	2.2	1.96
188	4.3	43.5	2	-	-	-	8.3	2.8	5	34.3	8.0	0.8	7.1	21.34
189	8.0	34.0	3	25.8	11.3	9	9.4	3.8	-	-	8.9	0.6	9.2	7.68
190	2.2	46.0	2	10.6	42.2	5	1.9	63.4	-	-	2.4	59.0	3.2	-1.92
191	4.0	34.0	3	13.8	23.1	2	5.9	57.2	-	-	-	-	4.0	8.21
192	2.5	36.7	1	16.9	29.8	5	7.0	9.1	6	36	8.2	6.7	6.6	15.00
193	8.2	48.0	2	4.7	47.5	2	8.3	20.8	8	27.6	6.9	17.6	6.3	27.15
194	5.7	48.0	3	-	-	-	9.0	0.0	-	-	8.5	0.0	8.8	41.28
195	5.8	32.3	2	33.4	0.0	8	8.9	4.5	8	19.9	8.9	0.2	8.5	18.43
196	3.5	28.3	4	-	-	-	0.9	54.2	-	-	-	-	1.0	53.81
197	3.7	43.0	2	-	-	-	4.8	26.6	4	43.4	4.8	25.0	4.5	26.51
198	7.8	35.3	4	-	-	-	8.0	5.9	-	-	8.5	0.5	8.3	59.84
199	13.8	33.5	2	5.4	56.1	1	1.0	53.5	-	-	1.3	59.4	1.1	13.35
200	6.0	25.5	5	12.4	37.1	7	-	-	-	-	-	-	7.0	4.29
201	7.4	25.8	1	10.2	55.0	2	1.8	65.0	1	68.9	1.0	57.5	1.5	52.15
202	7.0	39.0	2	8.3	39.1	2	3.4	51.2	-	-	-	-	2.8	19.19
203	-	-	-	27.7	12.7	8	-	-	-	-	-	-	8.0	58.69
204	3.8	36.6	6	2.6	43.2	3	2.5	47.6	-	-	1.8	68.4	2.4	31.17
205	12.0	30.3	5	-	-	-	3.4	66.8	-	-	-	-	3.5	44.93
206	3.0	35.3	1	-	-	-	7.8	19.9	7	30.7	8.3	14.0	7.7	38.31
207	9.5	29.0	2	19.1	21.0	6	8.9	5.3	-	-	-	-	7.5	5.13

Table C1. Continued

Experiment	RILs evaluation El Batan 2018			Test cross evaluation El Batan 2018			Test cross evaluation El Batan 2019		Test cross evaluation Obregon 2019		Test cross evaluation Toluca 2019		Combined Analysis	
RIL entry number	Sterile florets per spike	Seed per spike	Sterility visual score	Sterile florets per spike	Seed per spike	Sterility visual score	Sterility visual score	Seed per spike	Sterility visual score	Seed per spike	Sterility visual score	Seed per spike	Sterility visual score	Seed per spike
208	3.8	45.3	2	-	-	-	7.4	9.5	-	-	-	-	7.5	6.41
209	21.0	-	1	7.6	59.6	2	5.4	38.9	2	74.5	5.4	23.3	3.8	5.51
210	11.4	51.4	-	12.6	53.3	-	3.0	50.0	-	-	3.3	33.5	3.2	16.07
211	4.5	36.0	1	39.6	0.0	8	6.9	1.4	-	-	-	-	7.5	46.33
212	5.0	39.7	3	32.6	2.3	10	6.0	17.4	-	-	8.9	15.7	8.3	9.86
213	7.8	38.2	2	-	-	-	8.3	2.4	-	-	8.3	1.5	8.3	56.81
214	5.3	42.3	4	33.6	3.4	5	8.3	2.5	-	-	7.4	0.4	6.9	7.10
215	-	-	3	29.1	9.9	7	-	-	3	61.3	-	-	5.0	62.93
216	7.5	40.3	5	11.9	38.2	4	2.8	44.0	-	-	3.8	50.4	3.5	35.18
217	15.0	23.0	5	32.3	0.1	10	9.8	0.1	-	-	8.9	0.0	9.6	41.48
218	8.8	37.6	-	22.5	17.9	-	8.0	9.9	-	-	8.3	3.0	8.2	43.16
219	6.8	37.8	2	21.4	14.0	7	5.8	20.9	4	63.3	6.2	7.6	5.8	43.14
220	5.0	51.4	1	19.8	24.7	6	5.5	16.7	4	52.2	6.8	14.1	5.6	12.05
221	0.3	47.3	3	14.6	21.7	8	3.8	37.5	2	58.8	6.3	32.2	5.0	51.41
222	2.6	41.8	5	6.6	56.9	2	2.3	51.4	-	-	3.0	46.3	2.4	59.86
223	10.6	32.6	4	31.3	13.3	6	8.5	13.7	-	-	7.5	5.3	7.3	62.98
224	5.2	43.0	6	31.7	7.9	8	6.5	12.3	-	-	7.0	20.3	7.2	47.65
225	4.0	42.6	3	10.3	27.8	3	2.3	44.2	-	-	2.8	51.8	2.7	59.79
226	9.8	42.6	7	19.6	17.7	7	6.8	26.1	-	-	7.5	5.3	7.1	61.38
227	8.0	37.0	2	22.9	11.7	6	6.5	13.9	-	-	9.0	19.4	7.2	30.66
228	14.7	25.3	4	5.4	54.1	6	8.8	0.1	-	-	9.5	0.0	8.1	54.24
229	6.0	40.8	5	15.2	36.4	4	2.0	45.9	-	-	1.5	70.7	2.5	25.16
230	11.5	32.7	1	4.4	44.1	2	2.0	56.2	-	-	2.0	58.0	2.0	20.13
231	8.8	37.7	2	20.8	26.7	4	2.3	41.5	-	-	1.5	51.3	2.6	40.55
232	16.8	31.3	2	20.3	34.0	6	6.8	21.9	2	49.3	7.5	12.1	5.6	11.03
233	10.8	24.8	1	6.6	55.0	2	2.8	47.0	-	-	2.5	41.8	2.4	32.46
234	7.8	33.5	2	37.8	0.2	-	8.3	6.2	-	-	8.2	1.1	8.3	12.19

Table C1. Continued

Experiment	RILs evaluation El Batan 2018			Test cross evaluation El Batan 2018			Test cross evaluation El Batan 2019		Test cross evaluation Obregon 2019		Test cross evaluation Toluca 2019		Combined Analysis	
RIL entry number	Sterile florets per spike	Seed per spike	Sterility visual score	Sterile florets per spike	Seed per spike	Sterility visual score	Sterility visual score	Seed per spike	Sterility visual score	Seed per spike	Sterility visual score	Seed per spike	Sterility visual score	Seed per spike
235	9.6	29.8	4	-	-	-	9.3	2.3	-	-	10.0	0.3	9.7	42.86
236	23.4	25.0	1	-	-	-	5.8	27.3	2	57.4	6.8	18.4	4.9	38.37
237	10.2	46.4	-	5.8	67.4	-	3.9	35.5	2	64.1	4.5	14.9	3.5	3.94
238	2.4	40.4	3	8.8	41.2	5	1.8	48.0	-	-	1.5	55.7	2.8	42.54
239	5.8	45.3	7	7.9	40.8	2	3.4	62.0	3	66.8	-	-	2.8	50.69
240	15.8	29.5	7	-	-	-	9.0	0.1	-	-	8.5	0.0	8.8	11.48
241	4.0	35.5	1	35.3	0.0	8	1.4	51.9	2	61	1.9	43.9	3.4	37.02
242	5.5	33.5	2	-	-	-	1.4	55.3	1	52.6	1.9	75.2	1.5	26.75
243	9.0	17.3	3	11.0	33.8	7	3.8	46.0	-	-	4.0	48.2	4.9	6.28
244	10.0	35.4	1	33.4	0.3	9	8.2	2.5	-	33.8	8.5	0.6	8.6	16.83
245	4.5	41.0	2	-	-	-	5.4	23.7	-	-	-	-	5.5	56.51
246	3.8	58.2	1	21.0	29.1	6	1.8	67.4	-	-	2.3	67.7	3.4	13.93
247	23.2	32.0	-	7.6	51.9	3	4.8	14.4	-	39	1.8	73.8	3.2	54.34
248	12.0	23.4	4	21.9	24.2	7	6.0	25.3	-	-	7.4	20.0	6.8	10.54
249	6.0	29.5	4	20.2	11.5	8	-	-	-	-	-	-	8.0	16.35
250	3.6	50.7	1	10.9	28.5	5	6.5	9.6	4	40.6	8.0	4.5	5.9	16.81
251	8.8	43.0	2	-	-	-	8.5	3.4	-	-	8.8	0.8	8.7	19.70
252	5.7	42.7	2	31.6	18.7	-	5.8	14.7	-	38.2	5.5	19.6	5.7	3.91
253	15.4	32.5	3	41.1	0.0	-	8.8	0.0	-	1.2	9.0	0.0	8.9	28.03
254	16.0	21.3	6	-	-	-	8.3	10.1	-	-	9.0	17.0	8.7	13.53
255	12.8	24.8	-	13.0	35.6	3	8.8	1.3	-	-	9.3	0.6	7.0	10.38
256	16.2	25.0	5	10.8	26.7	2	4.4	32.2	-	-	4.9	35.5	3.8	25.80
257	5.2	38.0	2	21.3	16.4	6	6.5	4.2	-	-	7.8	2.3	6.8	54.38
258	15.2	25.8	4	19.2	17.8	5	5.3	8.5	-	-	8.5	18.9	6.3	41.39
259	6.0	26.8	3	33.5	0.3	8	8.0	1.8	-	-	8.8	2.0	8.3	30.38
260	7.6	41.0	3	-	-	-	9.0	2.2	-	-	9.3	0.6	9.2	26.67
261	5.5	27.5	1	19.6	30.9	5	2.3	55.6	-	-	3.3	60.0	3.5	49.69

Table C1. Continued

Experiment	RILs evaluation El Batan 2018			Test cross evaluation El Batan 2018			Test cross evaluation El Batan 2019		Test cross evaluation Obregon 2019		Test cross evaluation Toluca 2019		Combined Analysis	
RIL entry number	Sterile florets per spike	Seed per spike	Sterility visual score	Sterile florets per spike	Seed per spike	Sterility visual score	Sterility visual score	Seed per spike	Sterility visual score	Seed per spike	Sterility visual score	Seed per spike	Sterility visual score	Seed per spike
262	5.8	37.6	4	26.3	8.7	8	5.8	14.7	-	-	7.0	14.5	6.9	18.88
263	-	-	-	-	-	-	-	-	-	-	-	-	-	52.23
264	4.8	48.5	3	24.6	4.4	7	6.0	13.0	-	-	7.3	1.8	6.8	58.53
265	16.0	21.7	6	17.1	25.9	4	9.0	1.5	-	-	9.9	0.0	7.7	27.34
266	9.0	31.0	-	-	-	-	2.0	56.8	-	-	1.8	61.2	1.9	53.69
267	11.0	35.8	3	32.6	0.0	-	9.0	0.3	7	7.5	8.3	0.0	8.1	16.11
268	3.4	46.6	3	13.7	32.6	5	4.5	20.1	9	10.3	6.8	22.4	6.3	17.25
269	5.0	36.7	3	31.0	7.1	10	9.0	0.5	-	-	9.4	0.3	9.5	52.09
270	8.5	27.5	4	39.2	0.0	9	8.3	7.5	-	-	9.0	2.0	8.8	50.13
271	9.0	38.7	-	17.9	17.8	7	8.5	10.6	-	-	8.0	1.4	7.8	65.36
272	12.2	40.2	6	34.8	8.6	6	6.3	19.9	-	54.4	8.0	25.7	6.8	5.89
273	10.0	33.0	2	10.6	33.9	6	3.5	26.2	-	53.4	4.0	51.6	4.5	47.50
274	6.5	48.0	3	35.8	0.0	9	9.3	0.0	2	73.7	8.5	0.0	7.2	56.55
275	6.3	43.3	1	8.2	49.0	3	2.0	47.4	1	70.1	1.3	48.8	1.8	14.74
276	9.4	28.0	4	29.0	4.4	7	6.0	20.0	5	45	6.0	36.7	6.0	58.24
277	7.4	40.0	1	13.7	41.0	6	2.3	57.9	2	64.6	3.0	75.9	3.3	49.45
278	22.0	14.2	8	-	-	4	8.4	5.8	7	33	-	-	6.5	8.14
279	8.5	33.5	6	-	-	-	9.3	0.8	-	-	9.3	0.2	9.3	9.34
280	6.6	62.4	4	33.7	10.3	6	8.5	12.6	4	53	8.8	0.8	6.8	14.06
281	9.6	23.4	2	6.6	50.4	3	2.0	47.3	1	76.6	2.5	60.5	2.1	60.84
282	-	-	-	18.2	23.6	2	-	-	-	-	-	-	2.0	42.72
283	8.8	39.2	3	2.4	39.0	2	3.0	47.4	-	-	3.3	33.3	2.8	40.99
284	-	-	2	12.6	33.7	4	5.3	29.9	3	67.2	5.0	22.5	4.3	36.91
285	3.3	38.0	3	30.6	0.1	10	9.0	0.1	-	-	10.0	0.0	9.7	51.46
286	9.0	30.5	6	31.6	0.8	10	9.0	2.9	-	-	8.5	0.4	9.2	53.36
287	6.0	18.0	4	33.8	0.0	9	9.4	1.4	-	-	8.4	0.0	9.0	5.54
288	-	-	-	38.8	8.5	-	-	-	-	-	-	-	-	13.86

Table C1. Continued

Experiment	RILs evaluation El Batan 2018			Test cross evaluation El Batan 2018			Test cross evaluation El Batan 2019		Test cross evaluation Obregon 2019		Test cross evaluation Toluca 2019		Combined Analysis	
RIL entry number	Sterile florets per spike	Seed per spike	Sterility visual score	Sterile florets per spike	Seed per spike	Sterility visual score	Sterility visual score	Seed per spike	Sterility visual score	Seed per spike	Sterility visual score	Seed per spike	Sterility visual score	Seed per spike
289	2.5	39.8	4	16.0	29.3	2	2.3	42.9	1	66.6	1.8	46.5	1.8	3.55
290	5.3	32.5	8	-	-	-	2.5	54.5	-	-	2.5	51.6	2.5	40.55
291	3.8	42.2	3	26.8	1.9	9	7.5	3.2	-	-	8.0	1.0	8.2	57.03
292	8.0	37.3	2	3.1	51.3	2	1.5	56.0	1	69	1.0	75.4	1.4	49.01
293	9.8	52.5	2	5.4	43.2	5	6.0	22.7	-	60.4	8.3	14.4	6.4	55.41
294	0.8	39.0	3	17.4	17.7	6	2.3	54.5	-	-	2.8	37.1	3.7	-0.71
295	10.3	29.0	2	30.7	12.3	4	2.5	45.8	1	64.4	2.3	50.2	2.5	19.99
296	5.7	24.3	3	10.0	38.1	3	2.5	45.6	1	56	3.0	32.9	2.4	32.16
297	6.4	34.2	3	19.7	15.6	7	8.8	5.0	-	-	9.0	0.4	8.3	47.08
298	2.5	39.0	1	26.7	22.7	7	2.8	69.4	3	54.5	3.3	59.1	4.0	27.16
299	-	-	-	7.8	50.9	2	-	-	1	76.4	-	-	1.5	36.81

"-" Missing data

Table C2. Heritability estimates for traits used in mapping of fertility restoration. Phenotypic data came from six experiments conducted at three locations at El Batan, Obregon and Toluca, Mexico in 2018-19 for assessing fertility restoration in a recombinant inbred line (RILs) mapping population derived from a Cargill115 x C80 cross in International Maize and Wheat Improvement Center (CIMMYT), Mexico. Phenotypic data were collected from an experiment where RILs were evaluated and from four experiments in which test cross progenies from crosses between RILs and a cytoplamsic male sterile lines were evaluated for fertility restoration. Phenotypic data was collected on average seeds per spike, average sterile florets per spike and a visual score (1-10, 1 = fertile, 10 = sterile). Data for combined analysis for visual score came from five test cross evaluation experiments whereas combined analysis for seed per spike came from El Batan in 2018 and Obregon in 2019.

Year	Experiment	Trait	Heritability
2018	RILs phenotyping El Batan†	Sterile florets per spike	0.76
2018	RILs phenotyping El Batan	Seed per spike	0.74
2018	RILs phenotyping El Batan	Visual score	-
2018	Test cross phenotyping El Batan‡	Sterile florets per spike	0.94
2018	Test cross phenotyping El Batan	Seed per spike	0.94
2019	Test cross phenotyping Obregon	Seed per spike	
2019	Test cross phenotyping Obregon	Visual score	
2019	Test cross phenotyping El Batan	Visual score	0.96
2019	Test cross phenotyping El Batan	Seed per spike	0.95
2019	Test cross phenotyping Toluca	Visual score	0.97
2019	Test cross phenotyping Toluca	Seed per spike	0.95
2019	Combined analysis	Seed per spike	0.88
2019	Combined analysis	Visual score	0.93

†Data was collected from five spikes bagged to prevent cross-pollination before flowering.

"-" Only one set of observations were recorded.

‡Data was collected from ten spikes bagged to prevent cross-pollination before flowering.

Table C3. Summary statistics of genetic linkage map of recombinant inbred line population (Cargill115 x C80) constructed by using single nucleotide polymorphism (SNP) data from 20K genotyping array by TraitGenetics.

Linkage	Length (cM)	Number of markers	Marker density
1A1	75.66	112	0.68
1A2	46.49	51	0.91
1B1	168.12	284	0.59
1B2	24.67	74	0.33
1D1	53.89	23	2.34
1D2	47	32	1.47
2A1	110.94	148	0.75
2A2	118.47	55	2.15
2B1	86.22	48	1.80
2B2	66.97	115	0.58
2B3	39.29	30	1.31
2D	92.96	73	1.27
3A1	84.7	71	1.19
3A2	22.52	45	0.50
3B1	20.12	35	0.57
3B2	206.8	218	0.95
4A1	105.65	51	2.07
4A2	129.97	81	1.60
4B1	116.32	79	1.47
4B2	48.52	36	1.35
4D	43.61	21	2.08
5A1	24.34	26	0.94
5A2	117.78	234	0.50
5A3	41.37	27	1.53
5B1	48.31	66	0.73
5B2	174.57	268	0.65
5D	129.31	55	2.35
6A1	90.74	62	1.46
6A2	114.33	42	2.72
6B1	71.07	101	0.70
6B2	24.3	26	0.93
6D	28.38	25	1.14
7A1	165.7	112	1.48
7A2	80.2	67	1.20
7B1	99.56	153	0.65
7B2	58.47	76	0.77
7D	12.96	25	0.52
Total	2990.28	3047	0.98