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Perennial wheat lines have highly admixed population structure and elevated rates of outcrossing

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Abstract Perennial wheat has been proposed to alleviate long standing issues with soil erosion in annual cropping systems, while supporting rural communities and providing grain farmers with a marketable climate-resilient crop. The Washington State University perennial wheat breeding program has created several hundred interspecific progeny by crossing several different cultivars of winter wheat (*Triticum aestivum* L.) with *Thinopyrum* species and × *Agrotriticum* spp. Prior to the chromosome composition of these wheat-wheatgrass derivatives was not characterized, limiting their utility as stable breeding germplasm. We determined the mitotic chromosome number and species origin of chromosomes for eight breeding lines, and estimated their relatedness and population structure using AFLPs. Additionally, self-pollination and outcrossing rates were estimated for these breeding lines to gain an understanding of perennial wheat's reproductive strategy. We intercrossed the lines with each other to produce 20 families and then measured the level of chromosome pairing during meiosis I in the F_1 progeny. The lines contained between 44 and 64 chromosomes, of which eight to 16 were from *Th. ponticum*. Our analysis of molecular diversity indicated greater genetic diversity within, rather than across, breeding lines (88 and 12%, respectively). The outcrossing rate was estimated at 16%. Understanding chromosome number and origin is necessary for developing a population of breeding lines that can be used as parents. Our results suggest that the perennial wheat breeding lines act as a single diverse population that can be improved using breeding strategies for inbred and outcrossing crops.

Keywords Cytogenetics · Dryland agriculture · Perennial wheat · Population genetics · Wheat

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Introduction

Tillage-intensive, annual cropping systems are prone to extensive soil loss due to wind and water erosion

(Glover et al. 2010). After decades of intensive, dryland grain farming in the northwestern United States, soil loss has contributed to declining productivity (Busacca et al. 1985; Papendick et al. 1985; McCool and Busacca 1999). Cropping system options such as rotations with different cash crops or cover crops are limited in dryland farming systems that receive less than 300 mm of annual rainfall (Schillinger et al. 2006). A perennially growing wheat has been proposed as a solution for minimizing soil loss and improving other markers of soil health, sustaining rural communities, providing food security, and maximizing productivity from marginal lands (Bell et al. 2010; Glover et al. 2010; Crews et al. 2016; Crews and Rumsey 2017). In 1996, Washington State University (WSU) initiated a perennial wheat breeding program by crossing winter and spring varieties of hexaploid wheat (*Triticum aestivum* L.) with wild wheatgrasses from the *Thinopyrum* genus (Scheinost et al. 2001).

Both hexaploid wheat and *Thinopyrum* spp. are from the tribe Triticeae, a grass family whose members each have a haploid genome of 7 chromosomes (Löve 1984; Dewey 1984). However, many of those chromosomes have undergone numerous rearrangement and duplication events, resulting in speciation events due to different chromosome compositions and ploidy levels of Triticeae members (Barkworth and Dewey 1985; Maestra and Naranjo 1999; Luo et al. 2009; Hao et al. 2013; Wang and Lu 2014; Li et al. 2016). Interspecific and intergeneric crosses between Triticeae species are often attempted by researchers because of the similarity in chromosome composition and tolerance of F_1 progeny to aneuploidy (Sharma and Gill 1983a; Dewey 1984; Sharma 1995; Gill et al. 2006; Ceoloni et al. 2015). But, lack of pairing between the chromosomes of such wide crosses often results in sterile progeny. Wheat has frequently been crossed with wild relatives to capture small chromosomal segments that contain valuable alleles for crop production (Sharma and Gill 1983b; Friebe et al. 1996). Yet, both segments and whole chromosomes from wild species are often unwelcome in a crop improvement program because they carry alleles unfavorable for commercial production (Wulff and Moscou 2014) and can prevent chromosomal pairing and crossing over—an essential step in wheat breeding (Sears 1976).

Creating a perennial wheat that includes most or all of the wheat genome and a substantial portion of a genome from wild wheatgrasses is one approach of the WSU perennial wheat program. The program has created several hundred interspecific progeny by crossing several different cultivars of winter wheat with *Thinopyrum species* and × *Agrotriticum* spp. and has evaluated chromosome pairing in some of these hybrids. Prior to the study reported herein, we had not characterized the chromosome composition of these perennial wheat breeding lines. We also have yet to determine how many or which chromosomes or chromosomal segments are necessary to create a vigorous, high-yielding and high-quality perennial wheat variety. Lammer et al. (2004) found that a single chromosome addition from *Th. elongatum* to hexaploid wheat is sufficient for post-sexual cycle regrowth of the wheat-wheatgrass hybrid. Field trials of the WSU perennial wheat lines conducted in New South Wales, Australia indicated that a minimum of 56 chromosomes is required for post-harvest regrowth in derivatives of crosses between *T. aestivum* and *Thinopyrum* spp. (Hayes et al. 2012).

Crosses between *Thinopyrum* species and hexaploid wheat have been attempted frequently, often with successful results (Ceoloni et al. 2015). Crosses between *T. aestivum* ($2n = 6x = 42$) and *Th. ponticum* ($2n = 10x = 70$) have resulted in F_1 hybrids with 36–77 chromosomes per somatic cell (Dewey 1984; Jauhar 1995; Brasileiro-Vidal et al. 2005). Most of those hybrids had more than the total number of the wheat chromosomes (42). Derivatives beyond F_1 usually have between 42 and 44 chromosomes that are largely wheat chromosomes plus *Thinopyrum* chromosomes or translocations, likely due to a preference for limiting the introgression of wild genomic material (Friebe et al. 1996; Fedak and Han 2005). Derivates from crosses between wheat and *Th. intermedium* ($2n = 6x = 42$) have resulted in partial amphiploids with a chromosome number of 56 or greater (Banks et al. 1993; Cox et al. 2002; Han et al. 2004). Previous studies in wheat-*Thinopyrum* hybrids indicate that some chromosome pairing occurs during meiosis, particularly if *Ph1*, a locus suppressing homoeologous pairing is not expressed (Riley and Chapman 1958; Martinez-Perez et al. 2001). Cai and Jones (1997) observed that wheat and *Thinopyrum* chromosomes engage in autosyndetic pairing.

The reproductive strategy of perennial wheat lines is also not well understood at this time. A successful breeding strategy for perennial wheat will require a clear understanding of the reproductive strategy of the crop. Whereas hexaploid wheat cultivars are largely self-pollinated, many of the wild wheat *Thinopyrum* wheatgrasses reproduce primarily through outcrossing (Baumann et al. 2000). Most commonly, new wheat cultivars are developed by making a cross, driving cross progeny to homozygosity, and picking the best inbred (Allard 1999; Brown and Caligari 2008). In contrast, wild and semi-domesticated wheatgrasses outcross at a higher frequency than domesticated wheat due to a self-incompatibility mechanism (Baumann et al. 2000). Strategies for breeding outcrossing crops seek to maximize the combining ability between parents, thus masking deleterious alleles and avoiding inbreeding depression (Allard 1999; Brown and Caligari 2008). Genetically diverse outcrossing populations have been used for perennial wheatgrass cultivars (Dewey 1976; Knowles 1990; Asay 1992; Zhang et al. 2016).

To fill in some of these knowledge gaps in perennial wheat, the objectives of our study were to: (1) characterize the cytogenetics, genetic diversity, and population structure of selected WSU perennial wheat lines and (2) evaluate the reproductive strategy of these lines. Information about chromosome number and composition, in addition to regrowth characteristics, will further understanding of this germplasm as both cytogenetically stable perennial wheat and as advanced-generation progeny derived from wide hybridization. The degree of genetic heterogeneity of this germplasm and potential for traditional crossbreeding will also provide valuable information for evaluating the breeding strategies used in perennial wheat programs. Understanding population structure can illuminate how to optimally manage perennial wheat populations for breeding. Determining the extent of outcrossing, cross-fertility across breeding lines, and potential for outcrossing will enable breeders to leverage those features best for crop improvement.

Materials and methods

Plant material

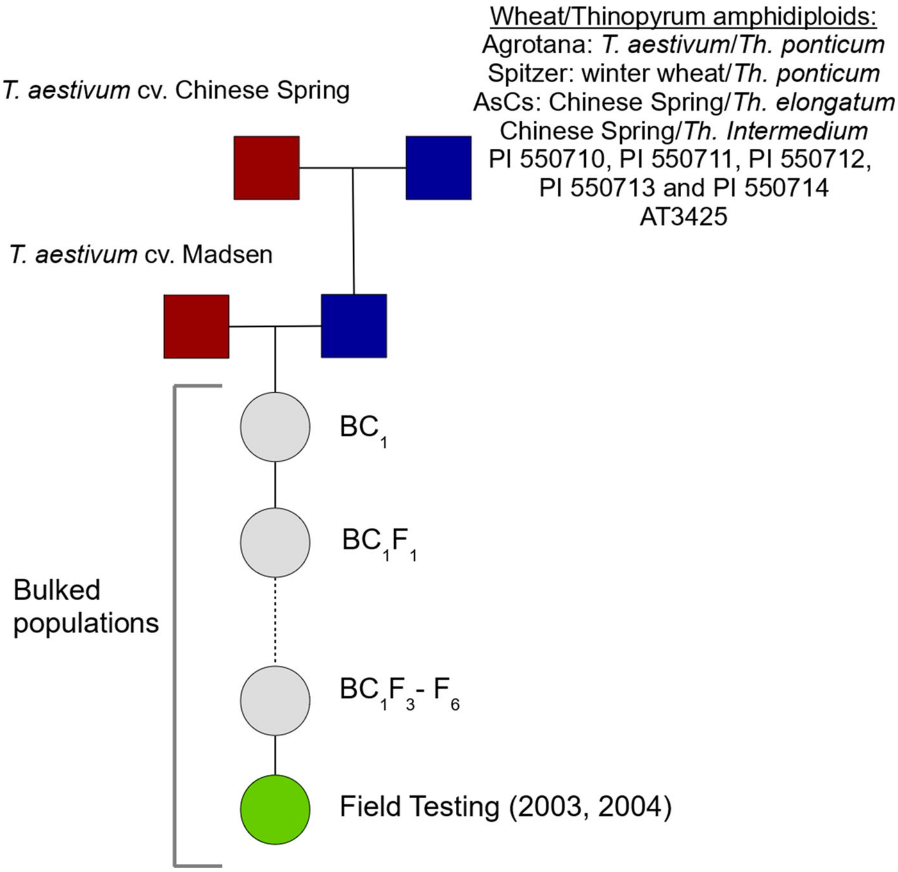
Eight WSU perennial wheat lines were selected as germplasm for cytogenetic analysis based on their morphological diversity and demonstrated ability to regrow after harvest and exhibit the perennial life habit. These lines included four evaluated during both the 2003 and 2004 field seasons, two evaluated during the 2003 field season, and two evaluated during the 2004 field season (Table 1).

The eight perennial wheat lines are derivatives of crosses between hexaploid wheat and *Thinopyrum* species, collectively termed × *Agrotriticum* spp. (Fig. 1). This term refers to all complete and partial amphiploids between the genera *Triticum* and *Agropyron*, some of whose members have been reassigned to the *Thinopyrum* genus (Curwen-McAdams et al. 2017). Although the complete pedigrees are unavailable, the basic outline of parents used and crosses performed is known. The pollen parents included in the initial crosses were the × *Agrotriticum* accessions PI 550713, PI 550714, and PI 550715; 'Agrotana' (*Th. ponticum*/hexaploid) (Chen et al. 1995); AgCs (*Th. elongatum*/Chinese Spring amphidiploid) (Cai et al. 2001); 'Spitzer' (winter wheat/*Th. ponticum*

Table 1 Perennial wheat breeding lines used in this study

Perennial wheat lines	Year(s) harvested	General morphological features
JP004	2003, 2004	Purple culm, elongated penducle, awned
JP016	2003, 2004	White culm, elongated penducle, awnless
JP019	2003, 2004	Purple culm, elongated penducle, awnless
JP023	2004	White culm, elongated penducle, awned
JP026	2003, 2004	White culm, elongated penducle, awned
JP028	2003	Dark culm, elongated penducle, awned
JP030	2004	White culm, elongated penducle, awned
JP034	2003	Gold culm, elongated penducle, awned

Fig. 1 Pedigree Schematic of the WSU Perennial Wheat Lines. Exact parentage, generations of selfing, and degree affected by seed mixing and misread map for each line and each individual is unknown



amphidiploid) (Oliver et al. 2007); and Chinese Spring/*Th. intermedium* partial amphiploid created by the WSU perennial wheat breeding program (Jones and Cai, unpublished data).

We crossed these eight lines to the wheat cultivar 'Chinese Spring' (CItr 14108). Viable progeny from those crosses were crossed at least once to 'Madsen' or 'Eltan', two Pacific Northwest-adapted winter wheat cultivars (Allan et al. 1989; Peterson et al. 1991). The F_1 were selfed for three to six generations, and the progeny from eight families were used in this study: JP004, JP016, JP019, JP023, JP026, JP028, JP030, and JP034. Of these eight families, individuals from four families from both the 2003 and 2004 field seasons were chosen for this study (JP004, JP016, JP019, and JP026). For the other lines, individuals were drawn from lines grown out during the 2003 field season (JP028 and JP034) and the 2004 field season (JP023 and JP030) (Table 1). Complete pedigree information for these families is unavailable due to insufficient knowledge of several × *Agrotriticum* spp. parent lines (Xu et al. 1994; Chen et al. 1995; Hang et al. 2005), incomplete record keeping, and seed contamination between the perennial wheat lines.

Two *Thinopyrum ponticum* accessions were used for crossing in this study, 'Russia' and 'Moore'. Both accessions were derived from single plants with high vigor and drought tolerance in a 200–250 mm annual rainfall zone in eastern Washington (Doug Lammer, pers. comm.).

Plant growth and propagation

All seeds were planted in potting soil and grown at 22 °C in a climate-controlled room with a 14-hour photoperiod for four weeks. Once they reached Zadoks growth stage 15 (Zadoks et al. 1974), the seedlings were vernalized at 4 °C with a 10-hour photoperiod for eight weeks. After vernalization, all plants were transplanted to 1-gallon pots and watered daily. The plants were fertilized with Osmocote fertilizer (14–14–14) one week after transplanting and supplemented with 0.5 grams of urea (40-0-0) as they initiated flowering (Zadoks growth stage 60).

Cytogenetics

We prepared chromosomes of somatic cells from young root tissue following the methods of Cai et al. (1996). Root tips were immersed briefly in ice-cold water for 24–28 h, and the chromosomes were fixed in Farmer's solution of 3:1 ethanol:acetate. Cells were squashed within one week of fixation on a glass slide and viewed on Zeiss Axioplan phase contrast microscope (Carl Zeiss, Oberkochen, Germany).

A total of 80 plants were analyzed for their somatic chromosome number. Pictures of cells were taken with a mounted digital camera and accompanying SPOT software (Diagnostic Instruments Inc., Sterling Heights, MI). All photos were examined with Adobe Photoshop Elements v4.0 (Adobe Systems Inc, San Jose, CA). Slides were frozen at −80 °C until further analysis.

We analyzed chromosome pairing in meiosis by preparing the chromosomes as described by Cai et al. (1996). Immature spikes were fixed in Carnoy's solution (1:3:6 chloroform:acetate:ethanol) for two to four days until they bleached white, and afterward were transferred to 70% ethanol. We examined pollen mother cells at metaphase I and anaphase I.

Genomic in situ hybridization was performed using the methods of Chen et al. (1999) with minor modifications. Sheared DNA from winter wheat was used as blocking DNA. Isolated DNA from *Th. ponticum* accessions Russia and Moore were the source of the probe. DNA was extracted with the CTAB protocol (Kidwell and Osborn 1992). We viewed the cells with the Zeiss Axioplan microscope using the appropriate filters for fluorescence.

Genetic diversity and population structure

We collected fresh, young leaf tissue from 3 individual plants each from lines JP004, JP016, JP019, JP026, JP028, and JP034 (all grown during the 2003 field season), and from Madsen, Eltan, Chinese Spring, and the *Th. ponticum* accession Russia. DNA was extracted from the frozen leaf tissue following a modified version of the Edwards et al. (1991) protocol. Using the sarkosyl DNA extraction method (Arterburn et al. 2011), DNA was extracted directly from individual seeds of lines JP004, JP016, JP023, JP026, and JP030, all grown during the 2004 field season.

The AFLP protocol used for the LI-COR DNA Analyzer (LI-COR Biosciences, Lincoln, NE) was modified from the Vos et al. (1995) protocol, which is described in Johnson et al. (2007). The samples were digested with *Eco*RI and *Mse*I and ligated with enzyme-specific adaptors. Pre-selective amplification was performed for 30 cycles of 94 °C for 30 s, 56 °C for 1 min, and 72 °C for 1 min. Selective amplification was performed using eight primer combinations, four labeled with IRD (infrared detection, LI-COR Biosciences) in the 700 nm range and four labeled with 800 nm IRD (Suppl. Table S2). Products were separated on polyacrylamide gel and migrating bands were detected by the LI-COR apparatus, viewed with Gene ImagIR 4.05 software (Scanalytics Inc, Fairfax, VA), and scored manually. Ambiguous bands were not scored.

Loci with a minor allele frequency of 5% or less were removed. Using the software I4A (Chybicki et al. 2011), we estimated the inbreeding coefficients for the perennial wheat breeding lines across the population and for each individual. Kinship was estimated using the method described by Hardy for dominant markers (2003) and implemented in SPAGeDi (Hardy and Vekemans 2002). The kinship values produced by SPAGeDi indicate relative relatedness rather the probability of identity by descent per se (SPAGeDI v1.5 User's manual, Sec. 6.1.1, 3 Mar 2015). The mean-centered kinship data were rescaled to a range of 0–1 by addition of a constant to all data points. Principal component analysis (PCA) was performed with R package Adegenet (Jombart 2008; Jombart and Ahmed 2011). Analysis of molecular variance (AMOVA) was completed for the breeding lines only (parental lines were excluded from the analysis) with the Poppr package v2.0 (Kamvar et al. 2014), using Nei's genetic distance as the distance function (Nei 1978). The number of populations was also estimated using the Poppr package and the software Structure (Falush et al. 2003). All R analyses were executed in version 3.3.0 (R Development Core Team 2011).

Reproductive strategy evaluation

The perennial lines were intermated using a controlled crossing scheme (Suppl. Table 1). The perennial wheat lines were also crossed with *Th. ponticum* accessions Russia and Moore and *T. aestivum* cv. Eltan. Developing embryos from crosses in which *Th. ponticum* was the egg donor were rescued approximately 14–18 days after pollination, following the William et al. (1987) protocol, and placed on MS basal salt media (Murashige and Skoog 1962) (Sigma Aldrich, St. Louis, MO).

For each cross, between 12 and 16 spikelets (two florets per spikelet) were emasculated at stigma maturity and pollinated with mature pollen. Seed setting from the crosses was expressed as number of developed seeds per emasculated florets within a spike. Chi square contingency tests were performed in R version 3.3.0 (R Development Core Team 2011).

To estimate seed setting potential by self-fertilization, spikes were covered prior to reproductive maturity. The total number of developed seeds were counted and expressed as a ratio relative to number of florets per spike. Only the two exterior and mature florets were used due the unpredictable seed development in the middle florets. Open-pollinated seed setting data were obtained in a similar manner, except that the spikes were not covered. We evaluated the seed setting results using a Chi square test with a 2 × 2 contingency table for the two possible types of fertilization: (1) self-pollination or (2) mixed self- and cross-pollination.

Results

Perennial wheat chromosome characterization

The chromosome number and composition of the perennial wheat lines varied extensively between and among breeding lines. A total of 202 cells were characterized; all exhibited a higher diploid chromosome number than hexaploid wheat, between 44 and 60 chromosomes (Table 2). Chromosomes from wheat comprised the majority of the genetic material, ranging from 28 to 42 chromosomes per cell. The number of *Thinopyrum*-derived chromosomes ranged from 8 to 12.

Genetic variation and relatedness

In the AFLP analysis, a total of 244 polymorphic markers were scored, ranging from 5 to 68 per primer pair (Suppl. Table S2). Forty polymorphic loci were removed for having a minor allele frequency less than 5%, effectively one allele across the individuals assayed. Of the remaining loci, 18 had identical alleles in all presumed parents included in this study: Chinese Spring, Madsen, Eltan, and *Th. Ponticum* (Fig. 2). Madsen and Chinese Spring had the highest number of shared alleles between them (76), while Madsen and *Th. Ponticum* the lowest number shared (39). *Th. Ponticum* had the largest number of unique alleles (36)

Table 2 Chromosome composition of the wheat-*Thinopyrum* amphidiploids from cytogenetic chromosomes counts and Genomic in situ Hybridization (GISH) of somatic cells

Perennial wheat lines	Chromosome origin		Total	n
	Th. ponticum	*T. aestivum*		
JP004 (2003)	12–14	30–42	48–52	3
JP004 (2004)	No data		56–62	5
JP016 (2003)	10–14	32–38	44–56	6
JP016 (2004)	12–14	34–48	54–64	12
JP019 (2003)	10–12	32–36	50–56	6
JP019 (2004)	14–16	36–38	54–56	12
JP023 (2004)	8–10	28–38	48–54	13
JP026 (2003)	No data		54–60	3
JP026 (2004)	10–14	36–42	54–56	8
JP028 (2003)	10–14	28–34	52–56	8
JP030 (2004)	12–14	30–38	50–54	9
JP034 (2003)	7–18	32–42	52–54	10

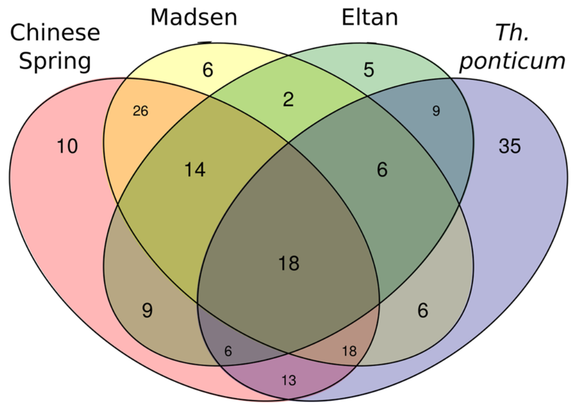


Fig. 2 Venn diagram of shared alleles among the presumed parents of the perennial wheat lines

and Eltan had the smallest number (5). AMOVA results for the within and between population genetic variation (excluding the *Th. ponticum* accession and the wheat cultivars for both analyses) indicated that most of the variation occurs between the individuals within lines rather than between lines. The among-individual variation was responsible for 88% of the total genetic variation and 73% of the genetic variation from the 2003-field season individuals. The remaining proportions, 12 and 27%, represents the between-line variation. The software Structure evaluated the posterior likelihood of the number of populations present in the individuals assayed. The log likelihood ratio tests evaluating the number of populations (from two to nine) compared to a null hypothesis of a single population indicates no statistically significant difference; hence, the level of admixture reflected a single population among the individuals assayed.

The kinship plot (Fig. 3) indicates low to moderate kinship between the individual lines, with a mean kinship of 0.35. Individuals with kinship values below the mean are less related than individuals drawn at random from the population. The perennial wheat lines demonstrated high kinship, which is typical of full sib derived families, but we observed inconsistent patterns across families. For example, the following families displayed higher kinship than others: JP004, JP016, and JP019 from field season 2003, and JP016 from field season 2004. We also found that kinship was higher for lines grown in a single year compared to lines grown across years 2003 and 2004. PCA did not indicate any additional population structure. The first three components explained 13, 12, and 10% of the variation, with decreasing significance in each principle component (data not shown).

Reproductive strategy

We observed a higher rate of seed set among inflorescences allowed to cross-pollinate compared to those restricted to self-pollination (Table 3). This phenomenon occurred in all lines except JP019 and JP034 grown in 2003 (JP034 lacked sufficient data for comparison). The difference in seed set across all individuals was statistically significant ($p < 0.001$) based on the Chi square contingency tests.

Intermated perennial wheat cross performance

Seventy-one crosses were attempted between the perennial wheat lines with an overall success rate of 9.8%. Of the progeny produced from these crosses, 88% were able to germinate and grow beyond the seedling stage. When these lines were grown to maturity, their rate of seed setting ranged from 0 to 79.2% (Table 3).

The majority of chromosomes paired during meiosis I of the F_1 intermated lines. The histogram of univalent distribution by frequency indicates that 54.8% of the cells examined (505 pollen mother cells) had two to four unpaired chromosomes per cell and 70.2% of the cells examined had four or fewer univalents (Fig. 4, Suppl. Table S1). Altogether, the number of univalents ranged from zero to 13. A pollen mother cell at meiotic metaphase I is shown in Suppl. Fig. S1. Post-senescence regrowth was observed in 51 out of 131 perennial wheat cross progeny (38.9%). All progeny that regrew following senescence also flowered after six weeks of vernalization.

Perennial wheat-Th. ponticum cross performance

A total of 78 progeny resulted from 71 different crosses between the perennial wheat lines and *Th. Ponticum* (Table 4). The overall success rate was 2.63% among all attempted crosses. Among the progeny, 66 seeds germinated (85% germination rate) and reached reproductive maturity. We found no statistically significant differences in cross success when different species served as the egg donor (Table 5). The *Th. ponticum* accession Russia had a higher seed setting rate than Moore. The rate of

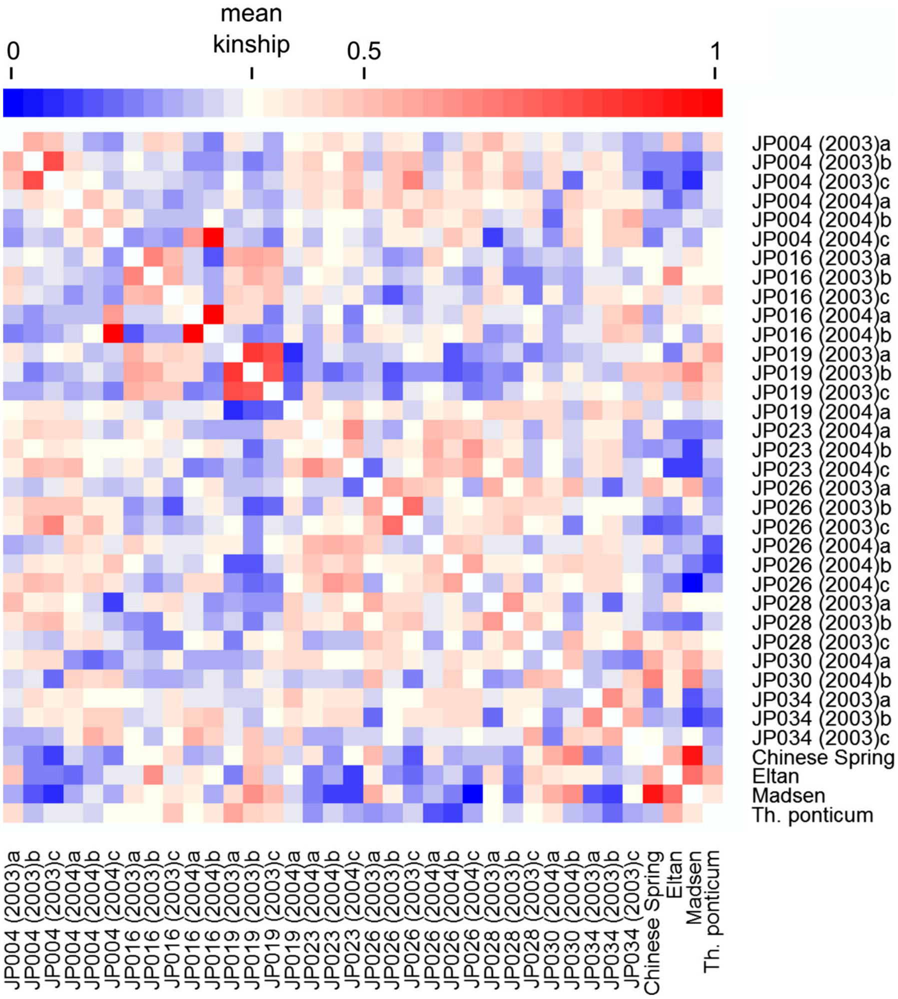


Fig. 3 Heatmap of the kinship coefficients between perennial wheat lines estimated from 209 AFLP markers. *Blue* and *red* colors indicate kinship below the average population kinship and above the population average, respectively

successful crosses was 2.1 times more likely when Russia was used as a parent. The success rate per cross combination (e.g., Moore/JP004) ranged from 1.0 to 53.8% (Suppl. Table S3). We observed a higher seed set when "Moore" was the pollen parent. Twenty-seven of the attempted crosses were unsuccessful, resulting in no viable seeds across the florets pollinated.

The somatic chromosome number of the F_1 perennial wheat-wheatgrass progeny ranged from 50 to 68. Twenty-one of the 26 lines with chromosome data had between 50 and 60 chromosomes (Suppl. Table S3, Suppl. Fig. S2). The variation in chromosome number of the F_1 progeny is due to the different chromosome number and composition of the parents. We also observed variation in the somatic chromosome number of the F_1 within cross families. This phenomenon can be attributed to chromosome loss in the parental gametes during meiosis. In all F_1 progeny, at least 50% of the florets of each individual produced seed.

Discussion

Prospects for stable reproduction

Although the perennial wheat lines appear to be undergoing stable reproduction marked by fertile offspring, the chromosome number and composition of each line and each individual clearly varies. These lines likely share many chromosomes since most of the chromosomes paired during meiosis of the F_1 progeny. Nevertheless, this study also found unpaired chromosomes in the F_1 progeny, which will most

Table 3 Seed set of perennial wheat amphidiploids under forced self-pollination only and self-pollination with cross pollinations permitted. The Chi square results indicate deviation from expectation of no cross-pollination

Lines	Seed set (%)		χ^2
	Self-pollinated inflorescences (%)	Selfed and outcrossing inflorescences (%)	
JP004 (2003)	72.64	83.44	14.88***
JP004 (2004)	52.17	61.54	2.31
JP016 (2003)	44.31	79.53	95.51***
JP016 (2004)	82.26	88.54	3.72
JP019 (2003)	63.21	57.59	1.03
JP019 (2004)	88.24	92.41	0.09
JP023 (2004)	86.27	92.77	0.096
JP026 (2003)	51.19	79.30	47.15***
JP026 (2004)	23.08	47.87	5.12*
JP028 (2003)	60.14	92.31	63.74***
JP030 (2004)	73.61	96.00	1.33
JP034 (2003)	No data	82.05	–
All lines	63.38	**79.92**	**157.2*** **

* Statistically significant at $\alpha = 0.05$

*** Statistically significant at $\alpha = 0.001$

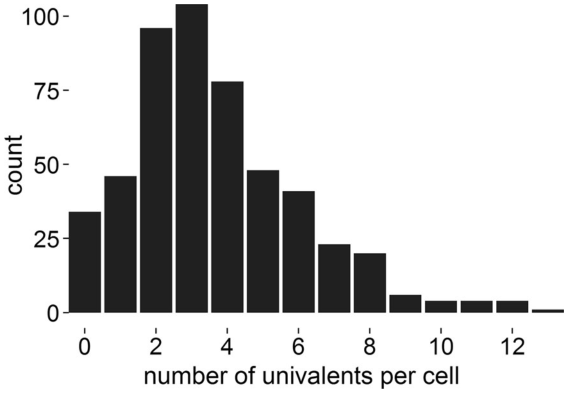


Fig. 4 Distribution of univalent chromosomes in the progeny of crosses between perennial wheat lines

Table 5 Results from Chi square contingency test

Effect	χ^2	*p* value
Th. Ponticum accession	11.69	<0.001
Egg donor for crosses with *Th. Ponticum*	2.62	0.11
Egg donor within 'Moore' crosses	11.53	<0.001
Egg donor within 'Russia' crosses	0.00	0.92

likely be lost in successive cycles of sexual recombination. Recombination and exchange of genetic material is hindered by unpaired chromosomes. Thus, the repeated occurrence of unpaired chromosomes in progeny from intermated perennial lines indicates that traditional breeding techniques exploiting

Table 4 Seed set of crosses between perennial wheat lines and *Th. ponticum* accessions 'Russia' and 'Moore', between the perennial wheat lines, and between the perennial wheat lines and *T. aestivum*

Type of cross	Total florets pollinated	Seeds produced	Seed set (%)
Russia/hybrid	796	29	3.52
Hybrid/Russia	846	30	3.55
Moore/hybrid	616	2	0.16
Hybrid/Moore	637	17	2.67
Interhybrid crosses	1947	199	9.8
Hybrid/*T. aestivum*	217	11	5.07

recombination between the individuals may be challenging. But, as the lines continue to intermate, an eventual chromosomal equilibrium—when all lines contain the same partial amphiploid chromosome complement—is possible.

No clearly defined population structure

The AFLP results indicate that the numbered lines assigned to the perennial wheat germplasm are not wholly consistent with the existing population structure in the germplasm. The perennial wheat breeding lines had a higher level of allelic diversity and a lower level of population differentiation than expected for an inbred crop. The number of polymorphic markers was higher than the 2–31 polymorphisms per primer pair reported for Pacific Northwest wheat germplasm (Barrett and Kidwell 1998) and higher than the 9–27 polymorphic bands for Argentinean wheat germplasm (Manifesto et al. 2001). Neither of the lines harvested in 2003 or 2004 included individuals with consistently higher kinship coefficients than expected if these lines were derived from a single bulked bi-parental cross family or the result of pure line selection. Additionally, the elevated level of between-population genetic variation is reflective of outcrossing species (Liu et al. 1998; Mable and Adam 2007). Together, these data show a lack of clearly defined population structure and suggest that the assigned numbers to the lines fail to accurately distinguish and describe the germplasm.

Reproductive strategy of perennial wheat

The higher seed set in perennial lines able to outcross and self-pollinate compared to self-pollination alone indicates: (1) a high proportion of shared chromosomes between the perennial wheat lines, and (2) evidence of elevated outcrossing rates compared to that of common wheat. Complete sterility is frequently a problem in the F_1 of intergeneric crosses between wheat and its relatives (Smith 1942). Because these lines were backcrossed to hexaploid wheat, we found increased chromosome pairing and concomitant improved reproductive fitness, as assessed by seed set rate. This high outcrossing rate among the perennial wheat lines likely occurred during field production of these lines, resulting in admixture between families.

The chromosome pairing patterns in the progeny of perennial wheat crosses provide further evidence of a high proportion of shared chromosomes among the perennial wheat lines. The relatively low number of univalents (averaging 3.67 per cell) compared to the total chromosomes in the cell (31 to 59), plus the high number of chromosome pairs (14–28), indicate a high proportion of shared chromosomes between the perennial wheat lines. Additionally, some of the parental chromosomes originated from *Th. intermedium* and *Th. Ponticum*. Although each of these progeny may have a varying chromosome composition due to different parents, many of them likely have overlapping, if not identical, chromosome composition. Two of the parents, AT 3425 and PI 550713, share the same *Thinopyrum* genome (Cai et al. 2001). Whereas the exact *Thinopyrum* chromosomes needed for perennial growth habit are unknown, chromosomes from different *Thinopyrum* genomes have conferred the perennial habit (Cai et al. 2001). *Thinopyrum* genomes are highly similar—having the same genome repeated—which has resulted in extensive sequence similarity, but with some structural differences due to chromosomal rearrangement (Moustakas 1993; Chen 2005; Wang et al. 2015; Zhang et al. 2016).

Implications for perennial wheat improvement

The majority of species in the Triticeae tribe have a self-incompatibility system which suppresses self-fertilization and hence prevents a pure-line breeding approach (Baumann et al. 2000). The self-incompatibility mechanism in the Triticeae is largely controlled by two independent loci (Klaas et al. 2011). The hypothesized evolutionary role of this mechanism is to maintain a rich menu of allelic diversity throughout a population for the long-term and prevent the loss of rare alleles (Charlesworth et al. 2005). Because these lines are derived from wide crosses with *Thinopyrum* spp., some individuals may also possess the self - incompatibility alleles.

Hexaploid wheat is largely managed as an inbred crop. However, the chromosome number variation, high allelic variation, and elevated outcrossing rate of these perennial wheat lines argue for incorporating genetic improvement strategies normally reserved for outcrossing species. Cox et al. (2002) argued for a recurrent selection scheme for domesticating wild wheatgrasses as well as the need to create populations with shared chromosomal composition in a perennial wheat improvement program. Given the chromosome composition, lack of population structure, and elevated outcrossing rate, these lines may be more accurately described as one large intermating population. Breeding strategies that rely on population-wide genetic diversity and leverage the natural outcrossing of a species may be more appropriate for this perennial wheat germplasm. Such strategies include bulking multiple selected individuals, hybrid breeding, composite cross bulk populations, synthetics, and open-pollinated varieties (Allard 1999; Jain 1961; Murphy et al. 2005), in addition to inbred strategies such as pedigree-based selection. A perennial wheat breeding program in Australia improved the average population performance by intermating the top performing lines for yield-related traits (Cox et al. 2010).

Promising gains have been made toward understanding grain yield over time, regrowth, and end-use quality of perennial wheat (Hayes et al. 2012; Murphy et al. 2009, 2010; Jaikumar et al. 2012; Gazza et al. 2016). In order to capitalize on those findings, we need to understand the number of different populations and families within a breeding program and the genetic variation within a germplasm pool. Our study results provide the first look into the reproductive strategy of perennial wheat and how to leverage that strategy for crop improvement. In addition to maximizing fecundity of perennial lines, intermating populations are also valuable because they enable new combinations of genomes, resulting in novel phenotypes. This reshuffling of valuable alleles can ultimately serve to improve perennial wheat.

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