Analysis of Genetic Diversity in Synthetic Wheat Assemblage (*T. turgidum*×*Aegilops tauschii*; $2n=6x=42$; AABBDD) for Winter Wheat Breeding

Uzma Hanif¹, Awais Rasheed², Alvina Gul Kazi^{1*}, Fakiha Afzal¹,
Maria Khalid¹, Muhammad Munir³ and Abdul Mujeeb-Kazi⁴

¹ Atta-ur-Rahman School of Applied Biosciences, National University of Sciences and Technology (NUST), Islamabad, Pakistan

² Crop Science Research Institute/National Wheat Improvement Centre, Chinese Academy of Agricultural Sciences (CAAS), Beijing, China

³ PMAS, Arid Agriculture University Rawalpindi, Pakistan

⁴ National Institute of Biotechnology and Genetic Engineering (NIBGE), Faisalabad, Pakistan

Received April 30, 2014; accepted May 28, 2014

Summary An unconventional genetic resource for bread wheat improvement is the diploid D genome donor to bread wheat; *Aegilops tauschii* ($2n=2x=14$, DD). Bridge crossing the *Triticum turgidum*/*Aegilops tauschii* results in synthetic hexaploid (SH) wheats; the most common method of wheat improvement that efficiently utilizes the diversity of this diploid wheat progenitor of the primary gene pool. SH wheats exhibit “spring” or “winter” growth habits, depending on the parental durum germplasm. This study focuses on the molecular diversity among 58 winter synthetic hexaploid wheat (WSH) combinations that have been produced to facilitate winter wheat breeding programs. Genetic diversity and genome-wide linkage disequilibrium (LD) were investigated for all D-genome chromosomes using SSR markers. LD analysis based on 71 microsatellite loci represented a mean marker density of 7.1 cm. A total of 738 alleles ranging from 3 to 20 per locus were characterized in WSHs. The mean PIC and MI values were 0.61 and 3.41, ranging from 0.14 to 0.86 and 0.02 to 9.6, respectively. The analysis of molecular variance and Wright’s fixation index further established significant genetic diversity among different clusters and durum groups leading to the conclusion that diversity within WSHs is well validated. The population structure also partially validated the cluster analysis by forming eight ($K=6$) sub-populations. The variable patterns of LD independent of the genetic distance (cm) in some cases provided critical information for these re-synthesized winter wheat genetic resources for their future deployment and association mapping studies. Molecular analyses, thus, have established ample diversity that is present within these winter SHs and generated information for effectively introducing new diversity from these unique genetic resources into the prevalent winter wheat germplasm.

Key words Winter synthetic hexaploid, SSR, Genetic diversity, *Aegilops tauschii*, *Triticum turgidum*, Linkage disequilibrium.

Winter wheat is a major crop in the developed world occupying a large proportion of the arable land in Europe and North America, with grain yields ranging from 2.5 to 8 t ha⁻¹ (FAO 2007). In developing countries, roughly 30% of the total of 117 million hectares is of facultative or winter growth habit (Dixon *et al.* 2009). The demand for wheat, based on production and stock changes, is expected to increase from the current level of approximately 625 million tons to around 813 million tons in 2030 and more than 900 million tons in 2050 (FAO 2007). Winter wheat

* Corresponding author’s e-mail: alvina_gul@yahoo.com

DOI: 10.1508/cytologia.79.485

productivity is crucial for contributing towards this production increase both in developed and developing countries. Winter wheat in Western Europe and NW USA contributes to global food security (Prasad *et al.* 2009). The high production of winter wheat grain in USA provides export grains for regions of the world which are not self sufficient in wheat grain like Africa and the Middle East. In the developing world the main area of winter and facultative wheat is in Western and Central Asia in those countries where rural poverty is widespread and wheat productivity increases are important to improve the livelihood of people (Dixon *et al.* 2001); that also includes China.

Genetic diversity is crucial for crop improvement strategies. The allelic diversity within the three gene pools of Triticeae is decisive for meeting the projected global demand of wheat (Rajaram 2001). The diversity within the D-genome of bread wheat is low as compared to the A- and B-genomes (Hao *et al.* 2011). Therefore, the key resources of variability for applied wheat improvement are the diploid D genome donor accessions of wheat; *Aegilops tauschii* (Trethowan and Mujeeb-Kazi 2008). Utilization of these genetic resources has developed germplasm that is readily useful for wheat improvement. Mujeeb-Kazi (2000) recommended that synthetic hexaploid wheats, produced by inter-specific hybridization of *T. turgidum* ($2n=4x=28$) and *Ae. tauschii* ($2n=2x=14$) followed by chromosome doubling, provided an effective mean to transfer genes across various ploidy levels, particularly from the wild diploid D progenitor *Ae. tauschii* accessions. Synthetic hexaploid wheats are now the recognized source of enhanced diversity for resistances/tolerances to many biotic and abiotic stresses (Mujeeb-Kazi *et al.* 2008), and the SH exploitation *via* bridge crossing also enriches the A- and B-genomes with its allelic variation.

Information on the amount of genetic variation in a crop is fundamental to its improvement, providing a basis for selection of superior parental combinations and prediction of progeny performance. The information can also help breeders to incorporate useful genetic variation into adapted gene pools by selecting loci linked marker alleles controlling favorable agronomic or quality traits (Tanksley and McCouch 1997). Several authors have studied genetic diversity in wheat by using molecular markers, such as microsatellites (Devos *et al.* 1995, Bohn *et al.* 1999, Donini *et al.* 2000, Manifesto *et al.* 2001, Christiansen *et al.* 2002, Huang *et al.* 2002, Dreisigacker *et al.* 2004, Fufa *et al.* 2005), amplified DNA fragment polymorphism markers (Manifesto *et al.* 2001), and randomly amplified polymorphic DNA markers (Joshi and Nguyen 1993). Microsatellite markers, also known as simple sequence repeats (SSRs), have proved to be the most suitable for studying wheat diversity because of their multi-allelic nature, chromosome and chromosome arm specificity, high polymorphism, and across genome distribution (Röder *et al.* 1998).

Multiple molecular techniques used to assess genetic diversity have been presented and compared in a review (Able *et al.* 2007). The genetic diversity studies have recently shifted to the linkage-disequilibrium (LD) analysis, or non-random association of alleles at adjacent loci within a population. This LD analysis is relatively more powerful to detect genetic diversity influenced by the population structure, historical (temporal and spatial) relationship among elite germplasm, genetic drift, directional selection, mating system, recombinational rates and gene fixation at different rates on different chromosome regions (Gaut and Long 2003). LD analysis has also facilitated the exploitation of genetic diversity in crops and is a basis for association mapping analysis. LD-based association studies have been conducted for various traits including kernel size, milling quality (Brescghello and Sorrels 2006), yellow pigment (Reimer *et al.* 2008), drought tolerance (Maccaferri *et al.* 2011) and late maturity α -amylase activity (Emebiri *et al.* 2010). In common wheat LD pattern has been assessed for chromosomes 2D, 5A (Brescghello and Sorrels 2006), and 3B (Horvath *et al.* 2009).

So far, genetic diversity within winter synthetic hexaploids developed at CIMMYT has not been documented. This study has focused on genetic diversity assessment among WSHs using SSR

Table 1. Pedigree of winter synthetic hexaploids used in this study.

WSH	Pedigree	WSH	Pedigree
1	Aisberg/ <i>Ae. squarrosa</i> (221)	30	UKR-OD 952.92/ <i>Ae. squarrosa</i> (304)
2	Aisberg/ <i>Ae. squarrosa</i> (310)	31	UKR-OD 952.92/ <i>Ae. squarrosa</i> (309)
3	Aisberg/ <i>Ae. squarrosa</i> (369)	32	UKR-OD 952.92/ <i>Ae. squarrosa</i> (311)
4	Aisberg/ <i>Ae. squarrosa</i> (446)	33	UKR-OD 952.92/ <i>Ae. squarrosa</i> (326)
5	Aisberg/ <i>Ae. squarrosa</i> (511)	34	UKR-OD 952.92/ <i>Ae. squarrosa</i> (358)
6	LEUC762.93/ <i>Ae. squarrosa</i> (409)	35	UKR-OD 952.92/ <i>Ae. squarrosa</i> (372)
7	LEUC762.93/ <i>Ae. squarrosa</i> (424)	36	UKR-OD 952.92/ <i>Ae. squarrosa</i> (409)
8	LEUC762.93/ <i>Ae. squarrosa</i> (1027)	37	UKR-OD 952.92/ <i>Ae. squarrosa</i> (428)
9	LEUC7846.93/ <i>Ae. squarrosa</i> (310)	38	UKR-OD 952.92/ <i>Ae. squarrosa</i> (511)
10	LEUC846.93/ <i>Ae. squarrosa</i> (409)	39	UKR-OD 952.92/ <i>Ae. squarrosa</i> (633)
11	LEUC846.93/ <i>Ae. squarrosa</i> (1024)	40	UKR-OD 952.92/ <i>Ae. squarrosa</i> (1018)
12	LEUC846.93/ <i>Ae. squarrosa</i> (1026)	41	UKR-OD 952.92/ <i>Ae. squarrosa</i> (1024)
13	UKR-OD 1169.91/ <i>Ae. squarrosa</i> (625)	42	UKR-OD 952.92/ <i>Ae. squarrosa</i> (1028)
14	UKR-OD 1169.91/ <i>Ae. squarrosa</i> (1024)	43	UKR-OD 952.92/ <i>Ae. squarrosa</i> (1031)
15	UKR-OD 761.93/ <i>Ae. squarrosa</i> (191)	44	UKR-OD 1530/ <i>Ae. squarrosa</i> (217)
16	UKR-OD 761.93/ <i>Ae. squarrosa</i> (192)	45	UKR-OD 1530/ <i>Ae. squarrosa</i> (306)
17	UKR-OD 761.93/ <i>Ae. squarrosa</i> (219)	46	UKR-OD 1530.94/ <i>Ae. squarrosa</i> (311)
18	UKR-OD 761.93/ <i>Ae. squarrosa</i> (392)	47	UKR-OD 1530.94/ <i>Ae. squarrosa</i> (310)
19	UKR-OD 1704.94/ <i>Ae. squarrosa</i> (1031)	48	UKR-OD 1530.94/ <i>Ae. squarrosa</i> (312)
20	UKR-OD 1704.94/ <i>Ae. squarrosa</i> (213)	49	UKR-OD 1530.94/ <i>Ae. squarrosa</i> (392)
21	UKR-OD 1704.94/ <i>Ae. squarrosa</i> (217)	50	UKR-OD 1530.94/ <i>Ae. squarrosa</i> (446)
22	UKR-OD 1704.94/ <i>Ae. squarrosa</i> (369)	51	UKR-OD 1530.94/ <i>Ae. squarrosa</i> (458)
23	UKR-OD 1704.94/ <i>Ae. squarrosa</i> (511)	52	UKR-OD 1530.94/ <i>Ae. squarrosa</i> (511)
24	UKR-OD 1871.94/ <i>Ae. squarrosa</i> (213)	53	UKR-OD 1530.94/ <i>Ae. squarrosa</i> (629)
25	UKR-OD 1871.94/ <i>Ae. squarrosa</i> (221)	54	UKR-OD 1530.94/ <i>Ae. squarrosa</i> (1024)
26	UKR-OD 1871.94/ <i>Ae. squarrosa</i> (323)	55	UKR-OD 1530.94/ <i>Ae. squarrosa</i> (1027)
27	UKR-OD 1871.94/ <i>Ae. squarrosa</i> (1024)	56	Pandur / <i>Ae. squarrosa</i> (223)
28	UKR-OD 1871.94/ <i>Ae. squarrosa</i> (1027)	57	Pandur / <i>Ae. squarrosa</i> (409)
29	UKR-OD 952.92/ <i>Ae. squarrosa</i> (188)	58	Pandur / <i>Ae. squarrosa</i> (515)

markers to assess linkage disequilibrium in this germplasm.

Materials and methods

Plant material

Genetic diversity was evaluated in 58 D genome derived winter synthetic hexaploid (WSH) wheats. The study was carried out at National Agricultural Research Center (NARC), Islamabad. The winter synthetic hexaploids were obtained by crossing nine winter-habit durum wheat cultivars with 31 *Ae. tauschii* accessions (Table 1). The F_1 hybrids ($2n=3x=21$, ABD) were treated with colchicine resulting in fertile synthetic hexaploid ($2n=6x=42$, AABBDD) wheats. This C-0 seed was increased and provided to the CIMMYT gene bank for storage and global distribution. A set was thus obtained for use in Pakistan.

Vernalization and cytology

All the synthetics were germinated in petri dishes. Root tips were collected for somatic cytology validation (Mujeeb-Kazi *et al.* 1994) from individual seedlings after which they were transplanted into jiffy peat pots. After one week, the seedlings were vernalized at 8°C for six weeks, then conditioned under ambient conditions in the growth room for 3 d (14 h light at 15,000 lux intensity and consistent temperature of 22°C), moved to the screen house with exposure to normal environmental conditions for a further 2 d hardening and finally transplanted in the field plots of NARC in Islamabad.

Table 2. Basic information about SSR markers used in diversity of winter synthetic hexaploids having higher grain weight.

S. No.	Locus/primer	TA	PIC	MI	S. No.	Locus/primer	TA	PIC	MI
1	<i>Xgwm33-1D</i>	8	0.68	4.17	37	<i>Xgwm609-4D</i>	11	0.56	6.31
2	<i>Xgwm106-1D</i>	5	0.64	2.05	38	<i>Xgwm624-4D</i>	10	0.7	1.26
3	<i>Xgwm232-1D</i>	11	0.43	4.73	39	<i>Xgwm642-4D</i>	11	0.67	0.84
4	<i>Xgwm337-1D</i>	13	0.8	9.60	40	<i>Xgwm16-5D</i>	10	0.63	0.26
5	<i>Xgwm147-1D</i>	9	0.53	4.77	41	<i>Xgwm121-5D</i>	15	0.38	0.63
6	<i>Xgwm642-1D</i>	8	0.74	1.89	42	<i>Xgwm174-5D</i>	12	0.83	2.95
7	<i>Xgwm30-2D</i>	18	0.5	6.25	43	<i>Xgwm182-5D</i>	12	0.38	6.46
8	<i>Xgwm102-2D</i>	11	0.53	7.81	44	<i>Xgwm190-5D</i>	8	0.6	1.88
9	<i>Xgwm157-2D</i>	6	0.74	4.44	45	<i>Xgwm469-5D</i>	5	0.61	1.10
10	<i>Xgwm210-2D</i>	3	0.17	0.51	46	<i>Xgwm205-5D</i>	11	0.14	0.10
11	<i>Xgwm249-2D</i>	11	0.81	8.91	47	<i>Xgwm212-5D</i>	6	0.71	1.89
12	<i>Xgwm261-2D</i>	3	0.63	1.89	48	<i>Xgwm269-5D</i>	5	0.58	1.86
13	<i>Xgwm296-2D</i>	4	0.4	1.60	49	<i>Xgwm271-5D</i>	10	0.63	0.25
14	<i>Xgwm320-2D</i>	6	0.56	0.46	50	<i>Xgwm272-5D</i>	7	0.69	2.46
15	<i>Xgwm349-2D</i>	10	0.7	7.20	51	<i>Xgwm292-5D</i>	5	0.79	3.95
16	<i>Xgwm455-2D</i>	3	0.66	1.98	52	<i>Xgwm358-5D</i>	16	0.38	0.02
17	<i>Xgwm484-2D</i>	8	0.72	3.24	53	<i>Xgwm565-5D</i>	7	0.86	3.07
18	<i>Xgwm515-2D</i>	2	0.38	0.76	54	<i>Xgwm583-5D</i>	10	0.5	0.10
19	<i>Xgwm539-2D</i>	11	0.72	8.11	55	<i>Xgwm654-5D</i>	9	0.61	1.02
20	<i>Xgwm608-2D</i>	11	0.48	6.24	56	<i>Xgwm55-6D</i>	6	0.78	4.68
21	<i>Xgwm2-3D</i>	11	0.82	9.02	57	<i>Xgwm325-6D</i>	8	0.68	1.36
22	<i>Xgwm3-3D</i>	10	0.64	4.10	58	<i>Xgwm469-6D</i>	12	0.6	0.18
23	<i>Xgwm52-3D</i>	12	0.68	4.59	59	<i>Xbacr204-6D</i>	11	0.68	7.48
24	<i>Xgwm71-3D</i>	7	0.76	5.32	60	<i>Xbarc5-6D</i>	10	0.82	13.17
25	<i>Xgwm161-3D</i>	3	0.51	0.68	61	<i>Xbarc173-6D</i>	9	0.48	0.80
26	<i>Xgwm183-3D</i>	6	0.59	3.54	62	<i>Xbarc183-6D</i>	10	0.74	7.40
27	<i>Xgwm314-3D</i>	3	0.65	0.87	63	<i>Xcfd132-6D</i>	12	0.69	4.66
28	<i>Xgwm341-3D</i>	11	0.44	3.67	64	<i>Xgwm37-7D</i>	12	0.44	0.15
29	<i>Xgwm383-3D</i>	8	0.7	1.40	65	<i>Xgwm111-7D</i>	9	0.67	3.65
30	<i>Xgwm456-3D</i>	6	0.48	1.28	66	<i>Xgwm121-7D</i>	10	0.38	0.56
31	<i>Xgwm497-3D</i>	7	0.58	2.98	67	<i>Xgwm295-7D</i>	11	0.61	0.39
32	<i>Xgwm664-3D</i>	6	0.76	4.56	68	<i>Xgwm350-7D</i>	10	0.63	0.47
33	<i>Xgwm645-3D</i>	9	0.79	7.11	69	<i>Xgwm428-7D</i>	8	0.34	0.24
34	<i>Xgwm165-4D</i>	8	0.68	5.44	70	<i>Xgwm437-7D</i>	11	0.51	0.54
35	<i>Xgwm194-4D</i>	8	0.83	3.74	71	<i>Xgwm635-7D</i>	4	0.81	0.93
36	<i>Xgwm608-4D</i>	3	0.36	1.08					

TA=total alleles; PIC=polymorphic information content; MI=marker index.

The previously collected root tips from individual seedlings of each synthetic seedling were cytologically validated and at least five seedlings per entry with the euploid number of $2n=6x=42$ were identified to be planted in pots for a pure seed increase and maintenance while the remainder were subjected for phenotypic evaluation.

Molecular evaluation

In total, 71 SSR D genome specific primers were used for screening the winter synthetic germplasm (Table 2). DNA was extracted from the euploid potted plants of each WSH entry using methods adapted from Rogowsky *et al.* (1991). In general, a PCR protocol according to Röder *et al.* (1998) with 35 cycles of 1 min 94°C denaturation, 1 min 50°C (or 55°C or 60°C depending on the primer) annealing and 1 min of 72°C extension followed by a final extension step of 5 min 72°C was used. T-CY CreaCon PCR systems were used for all amplification reactions.

For electrophoresis of the amplification products, 2 g high resolution agarose/2 g agarose/TBE

gel were used. Gels were visualized by ethidium bromide under the UV light chamber and observed using the computer program UVIPhotoMW. Dolphin 1D image analysis software version 1.1 was used to compare and score the generated bands.

Statistical analysis

Molecular

Microsatellite loci were scored individually and alleles recorded as 1 for presence and 0 for absence. Genetic similarities between WSHs were calculated with the Dice coefficient based on the proportion of shared alleles using NTSYS-pc version 2.1 (Rohlf 2000). Cluster analysis was performed based on similarity matrices with the Neighbour-Joining (NJ) method, and the dendrogram constructed by importing the similarity matrices to DarWIN 5.0. levels of genetic variation within and among five clusters as identified by cluster analysis and nine durum groups (genotypes with same durum parent were grouped) were estimated from the allelic frequencies using the analysis of molecular variance (AMOVA) (Weir and Cockerham 1984). Implementation utilized the software Power Marker V. 3.25. These analyses separated the total molecular variance into components of inter- and intra-groups and tested among group significance levels. Wright's fixation index (*Fst*) estimated the variance within groups, with statistical significance of the 'index' evaluated by using a 1000-times permutation of the genotypes among and within groups. Genetic diversity among the clusters was evaluated based on the pair-wise *Fst* comparison that measures the allelic fixation in the population.

Polymorphism information content (PIC) assessed the genetic diversity at each locus. P =the number of polymorphic loci/the number of all loci. PIC was calculated as described by Anderson *et al.* (1993). $PIC=1-\sum P_{ij}^2$, where relative frequency of the *j*th allele for the *i*th locus was summed across all the alleles for the locus over all lines. PIC provides an estimate of the discriminatory power of a locus by taking into account, not only the number of alleles that are expressed, but also the relative frequencies of those alleles. PIC values range from 0 (mono-morphic) to 1 (very highly discriminative) with many alleles in equal frequencies. Senior *et al.* (1998) opined that PIC is synonymous with the term 'gene diversity' as described by Weir (1996). Marker index was calculated for each SSR primer as $MI=PIC \times \eta \beta$, where PIC is the mean PIC value, η the number of bands, and β is the proportion of polymorphic bands (Table 2) (Powell *et al.* 1996). The patterns of genetic variation among varieties were analyzed using Nei's measure of genetic diversity, which for each locus was calculated as $n(1-\sum P_{ij}^2)/(n-1)$ where P_{ij}^2 is frequency of the *j*th allele for the *i*th locus summed across all alleles (*n*) in the locus. Gene diversity of genotypes is the average diversity of all loci (Nei 1987).

Population structure

Population structure was estimated with unlinked markers using STRUCTURE 2.3.3; a model-based (Bayesian) cluster software (Pritchard 2000). The number of subpopulations (*K*) was set from 2–20 based on admixture and correlated allele frequencies models. For each *K*, 10 runs were performed separately. Each run was carried out with 30,000 iterations and 30,000 burn-in periods. A value of *K* was selected where the graph of $\ln Pr(X/K)$ peaked in the range of 2–20 subpopulation. For selected *K*, again, 10 runs were performed each with 100,000 iterations and 100,000 burn-in periods.

LD analysis

Pairwise linkage disequilibrium pattern was measured using TASSEL 2.0.1 software (Zhang *et al.* 2006). The comparison-wise significance was computed using 1000 permutations as implemented in TASSEL software. Since SSRs are multi-allelic markers, a weighted average is suggested to estimate r^2 values (Farnir *et al.* 2000, Horvath *et al.* 2009). Therefore in this study, the

Table 3. Number of markers, number of alleles per locus and average diversity at each D-genome chromosome investigated across 58 WSHs.

Chromosome	Number of markers	Number of alleles per locus	Average diversity $\pm$ SE*	Average MI $\pm$ SE	Average LD $\pm$ SE
1D	6	9.0	0.63 $\pm$ 0.05	4.535 $\pm$ 1.14	0.05 $\pm$ 0.02
2D	14	7.86	0.57 $\pm$ 0.04	4.24 $\pm$ 0.86	0.06 $\pm$ 0.008
3D	13	7.33	0.64 $\pm$ 0.03	3.77 $\pm$ 0.68	0.03 $\pm$ 0.006
4D	6	10.66	0.63 $\pm$ 0.06	3.11 $\pm$ 0.97	0.02 $\pm$ 0.007
5D	16	9.2	0.58 $\pm$ 0.04	1.34 $\pm$ 0.31	0.04 $\pm$ 0.007
6D	8	9.75	0.69 $\pm$ 0.03	4.96 $\pm$ 1.51	0.02 $\pm$ 0.004
7D	8	9.37	0.54 $\pm$ 0.05	1.93 $\pm$ 0.51	0.05 $\pm$ 0.013
Overall mean	(Total: 71)	9.02 $\pm$ 1.12	0.61 $\pm$ 0.05	3.41 $\pm$ 1.35	0.04 $\pm$ 0.01

* SE, Standard error.

r^2 correlation index between alleles at loci was chosen as the index (Horvath *et al.* 2009). The position of SSR loci in terms of genetic distances (cm) was based on the Ta-SSR-2004 consensus map (Somers *et al.* 2004). The CMap Matrix tool (<http://www.geneica.net.au/index.php?title=CMap>) was then used for the SSR loci for which gene map position was not available. For such markers, position was aligned by comparing the Ta-SSR-2004 map with either the wheat composite 2004 map or the Ta-Synthetic $\times$ Opata-SSR map, following the details described by Chao *et al.* (2007). LD levels and the rate of LD decay were computed by calculating r^2 for pairs of SSRs and plotting them against genetic distance. The statistical significance of individual r^2 estimates was calculated by the exact test following Weir (1996). Chromosome specific r^2 values were plotted using the R package LDheatmap (Shin *et al.* 2006).

Results

Molecular evaluation

The number of alleles amplified, number of polymorphic alleles, PIC values and Marker Index (MI) for each SSR marker are given in Table 2. The maximum number of alleles (13) was amplified by *Xgwm337-1D* with six SSRs amplifying only one allele. A total of 612 alleles were amplified in these WSHs with a mean of 8.61 alleles per primer pair. The maximum number of average alleles (10.66) was observed for chromosome 4D followed by 6D (9.75) (Table 3). PIC values ranged from 0.14 (*Xgwm205-5D*) to 0.86 (*Xgwm565-5D*) with 0.61 being the average. The maximum PIC value (0.69) was observed for chromosome 6D followed by 3D (0.64) which is more reliable due to the number of markers (13) specific to 3D (Table 3). Marker index (MI) was also calculated for each SSR primer pair and ranged from 0.02 (*Xgwm385-5D*) to 9.6 (*Xgwm337-1D*) with an average of 3.41. MI is an important parameter, which takes into account the markers' efficiency to amplify more alleles, and is an important comparative criterion when two markers have the same PIC value (see formula). MI was the highest (4.96) for chromosome 6D followed by 1D (4.53) and lowest (1.34) for chromosome 5D. The higher values of alleles per locus, PIC and MI indicate maximum variability and allelic richness residing within chromosome 4D. The maximum number of score-able bands (654) was obtained with the primer *Xgwm337-1D* (data not shown). Different primer pairs showed variation in their ability to detect polymorphism.

SSR amplification data was used to generate a similarity matrix for estimating genetic diversity and relatedness among the 58 synthesized winter synthetics. The value of similarity coefficient of WSHs ranged from 0.646 (64.6%) to 0.906 (90.6%). The average similarity coefficient value was 0.863 (86.3%) and hence a 13.2% genetic distance was observed.

Using the NJ Dice coefficient the cluster analysis separated the 58 WSHs into six main

Fig. 1. Neighbour-Joining dendrogram. a) Dendrogram of 58 WSHs based on 71 SSR markers using Dice coefficient (accessions within same group are marked with same sign). b) Nine durum groups of 58 WSHs based on 71 SSR markers using Nei's genetic distance algorithm (values in parentheses are number of WSHs within that durum group).

clusters (Fig. 1). The separation of genotypes was independent of the pedigrees as the A- and B-genome specific markers were not taken into account. Nine durum groups were made by combining the WSHs possessing the same durum parent. WSHs derived from durums UKR-OD 1704.94 and UKR-OD1871.94 were placed in the same group due to the genetic similarity of these sister lines (durums). Similarly, durum genotypes LEUC762.93 and LEUC846.93 are also sister lines. WSHs derived from UKR-OD1530 and UKR-OD1530.94 were grouped separately due to their different genetic background (Toma Payne, personal communication). Cluster analysis of these nine durum groups was obtained (Fig. 2) which separated the Ukrainian-derived durum from

Fig. 2. a) Estimated population structure. Each individual is represented by a thin vertical line, which is partitioned into $K=6$ segments that represent the individual's estimated membership fractions (%) in K . b) Plot of the average logarithm of the probability of data likelihood, ($\ln P(D)$), as a function of the number of assumed subgroups (K), with K allowed to range from 2 to 20.

Table 4. Analysis of molecular variance (AMOVA) for the six clusters of 58 WSHs identified in Fig. 1 using Dice coefficient.

Source	df	SS	Est. Var.	% of variation	<i>p</i> -Value
Among clusters	5	36.25	0.161	4.27	<0.001
Among WSHs	52	223.2	0.685	18.18	<0.001
Within WSHs	57	169.5	2.922	77.55	<0.001
Total	114	428.97	3.768	100	

that of Aisberg and Pandur thereby indicating sufficient variability within each durum derived synthetic. Both clusters were then subjected to AMOVA. The distribution of molecular variance among and within the identified clusters in Fig. 1 is described in Table 4. The AMOVA revealed that 4.27% of the total variation resided among the clusters, 19.07% among WSHs within the clusters whereas the remaining 77.85% diversity resided within WSHs. Similarly for the durum groups ($n=9$) the AMOVA estimate of 3.08% of the total variability resided among durum groups, 19.07% among WSHs within durum groups and 77.85% of the total variability was depicted within WSHs (Table 5). By comparing both AMOVA procedures, it was evident that the clusters identified in Fig. 1, as compared to durum groups, explained better genetic variability.

To have a better understanding of the genetic diversity between each durum group (Fig. 2) and clusters (Fig. 1), pair-wise *F_{st}* values were computed. Pair-wise Wright's fixation index (*F_{st}*) between all durum groups revealed significant differences between D1–D5 (0.063), D1–D6 (0.09),

Table 5. Analysis of molecular variance (AMOVA) for the nine durum groups of 58 WSHs using Nei's genetic distance.

Source	df	SS	Est. Var.	% of variation	<i>p</i> -Value
Among durum groups	8	46.12	0.116	3.08	<0.001
Among WSHs	49	213.34	0.716	19.07	<0.001
Within WSHs	58	169.5	2.922	77.85	<0.001
Total	115	428.97	3.754	100	

Table 6. Pair-wise Wright's fixation index (*F_{st}*) values between the six clusters of 58 WSHs identified in Fig. 1.

Cluster	<i>n</i>	1	2	3	4	5
1	16	—				
2	11	0.014	—			
3	15	0.079**	0.075**	—		
4	6	0.068**	0.046*	0.020	—	
5	6	0.041*	0.031*	0.020*	0.003	—
6	4	0.009	0.003	0.149**	0.118*	0.065*

p*<0.05; *p*<0.01.**Table 7.** Pair-wise Wright's fixation index (*F_{st}*) values between the nine durum groups as identified in Fig. 2.

Durum group	<i>n</i>	D1	D2	D3	D4	D5	D6	D7	D8
Aisberg (D1)	5	—							
LEUC (D2)	7	0.024	—						
UKR-OD 1169.91 (D3)	2	0.086	0.070	—					
UKR-OD 761.93 (D4)	4	0.040	0.034	0.004	—				
UKR-OD1704.94 (D5)	10	0.063*	0.008	0.069	0.017	—			
UKR-OD952.92 (D6)	15	0.090*	0.040*	0.012	0.051	0.030	—		
UKR-OD1530 (D7)	2	0.027	0.097	0.180*	0.171*	0.130*	0.026*	—	
UKR-OD1530.94 (D8)	10	0.086**	0.016	0.059	0.005	0.007	0.017	0.018	—
Pandur (D9)	3	0.044	0.038	0.007	0.036	0.032	0.062	0.346*	0.003

p*<0.05; *p*<0.01.

D3–D7 (0.18), and D6–D7 (0.026) pairs at *p*<0.05, while at *p*<0.01 D1–D9 (0.086) differed significantly (Table 7). Similarly for the clusters in Fig. 1, Cluster 1–3 (0.079), Cluster 1–4 (0.068), Cluster 2–3 (0.075) and Cluster 4–6 (0.149) were significantly different at *p*<0.01 and Cluster 1–5 (0.041), Cluster 2–4 (0.046), Cluster 2–5 (0.031), Cluster 3–5 (0.020) and Cluster 4–6 (0.118) were significantly different at *p*<0.05 (Table 6). *F_{st}* values for both groups were almost the same; however between clusters more values were significantly different as compared to the durum groups.

Population structure

Population structure of all 58 WSHs was investigated using a Bayesian clustering approach, to infer the number of clusters (populations) with STRUCTURE v2.2 software. The structure result at *K*=6 (Fig. 2a) was the best separator providing the highest Δk value (Fig. 2b). The structure results were consistent with the cluster analysis. The other implications of the structure of population have been described above.

Fig. 3. Pairwise LD-plots based on wheat consensus map showing distribution of LD (r^2) patterns in chromosome a) 1D, b) 2D, c) 3D, d) 4D, e) 5D, f) 6D and g) 7D.

LD analysis on D-genome chromosomal scale

In the present study 71 SSR loci with a mean marker density of 7.1 cm per locus were used to measure LD for all D genome chromosomes. The extent of chromosome-wise LD values is appended in Table 3. Since allele suppression/expressions is a widely observed phenomenon in synthetic hexaploids (Bai and Knott 1992, Ma *et al.* 1995), all winter synthetics were taken into account for these analyses, since there were several similar *Ae. tauschii* accessions utilized with different durum cultivars (like WSH-6 and WSH-10). Pairwise LD plots for all D-genome chromosomes scaled on wheat consensus map are given (Fig. 4). Maximum pairwise LD (0.06)

Fig. 4. LD decay among SSR marker pairs as a function of genetic distance (cm) in D-genome chromosomes.

was observed in chromosome 2D followed by 1D (0.05) with an average of 0.04 for all D-genome chromosomes. The maximum number of non-significant pairwise LD values was observed for the markers specific to chromosome 1D. The regions of significant LD that involved loci 10 cm or less apart were present for most of the chromosomes, except 4D and 7D. The exceptions where unlinked loci have significant LD estimates were detected in chromosome 2D, 4D and 7D. In contrast, several loci spaced less than 5 cm apart did not exhibit strong LD such as chromosomes 3D and 5D. The extent of LD decay as a function of genetic distance (cm) was further evaluated for all chromosomes (Fig. 3). The extent of LD decay varied greatly across the chromosomes, even for the closely linked loci. This variability was comparatively greater for chromosome 5D.

Discussion

Winter wheat production significantly contributes to global food security that like spring wheat is influenced by harnessing the genetic diversity resources of the family *Triticeae*, where *Aegilops tauschii* plays a significant role through synthetic wheat exploitation. Genetic diversity present within winter synthetic hexaploids (WSHs) can add value by introducing new allelic variation into products where breeding and intensive selection pressure have reduced allelic diversity, causing major constraints for winter wheat improvement emerged within the modern agriculture system (Prasad *et al.* 2009, Karsai *et al.* 2012). The challenges faced by winter wheat breeders in the Great Plains (the largest winter wheat producing area in the USA) include moderate to high incidence of aphids, green bugs and Russian wheat aphids. Similarly, priority breeding objectives for European winter wheat breeding programs include resistance to powdery mildew, stripe rust, industrial quality and resistance to other biotic stresses (Karsai *et al.* 2012). The genetic diversity present within synthetic hexaploids has been assessed for its resistance to Russian wheat aphid (Castro *et al.* 2004, Sotelo *et al.* 2009), green bug (Lage *et al.* 2003, Castro *et al.* 2004), and powdery mildew (Gill *et al.* 1996, Lutz *et al.* 1995, Miranda *et al.* 2006). The diversity is expansive and highly pursued by global wheat researchers using conventional and spring wheat based alien genetic resources (Mujeeb-Kazi 2003, 2006, Mujeeb-Kazi *et al.* 2008). This diversity in a limited manner as to quantity has now been placed in winter germplasm through WSHs, thus adding efficiency to the exploitation of the D genome relative for winter wheat improvement. In maintenance of the synthetics it is advisable that normalcy be maintained for critical research as

encountering aneuploidy does exist. Both hyper- and hypo-ploidy were observed when somatic counts were made to identify at least four individual seedlings per entry for potted plant increase that possessed $2n=6x=42$ chromosomes. Seeds produced from these euploid plants are to serve for further studies that require germplasm integrity precision. Though differential staining was not done to validate the chromosomal constitution, meiotic analyses across all WSHs did show perfect bivalency at metaphase I (unpublished data).

Molecular diversity based on SSRs

Narrowing this number involves unraveling the DNA profile for its polymorphic diversity through diagnosis *via* SSRs that are chromosome arm and genome specific (Röder *et al.* 1998). In some experiments hundreds of markers were applied to cover the genome leading to genome wide association through linkage disequilibrium analysis (Chao *et al.* 2007). However a much lesser marker number is sufficient for analyzing genetic diversity in germplasm collections. The earlier reports of genetic diversity of European wheat accessions were shown by Röder *et al.* (2002), who used 19 SSR markers. A vast amount of information is available on the genetic diversity that existed in European and worldwide wheat germplasm collections, especially landraces (Huang *et al.* 2002, Roussel *et al.* 2005, Balfourier *et al.* 2007). In case of winter wheat cultivars and landraces, recent reports of Karsai *et al.* (2012) and Parsad *et al.* (2009) found sufficient genetic diversity within these pools. However, new resources need to be incorporated in order to get a maximized genetic base for improving winter wheats. New resources are paramount for enriching the wheat gene pool and upon targeted exploitation provide value to the crop to exhibit durable resistance in order to sustain yields and address wheat-based food security.

In our knowledge this study is the first report on elucidating the genetic diversity of winter wheat synthetic hexaploids by applying molecular markers. The markers demonstrated that significant genetic diversity resided in WSHs and was contributed by *Ae. tauschii* with additive value coming from the A and B genomes of the durum wheat cultivars. SSR markers analyzed revealed a genetic diversity of 0.61 and mean allele number of 10.64 over 71 loci for this winter synthetic sub-set. These values are relatively higher than previously reported estimates of 7.2 alleles among 43 US wheat germplasm over 242 loci (Chao *et al.* 2007), 6.2 alleles among 40 elite European cultivars over 23 loci (Plaschke *et al.* 1995), 5.4 alleles and gene diversity of 0.57 among 68 advanced lines from CIMMYT over 47 loci (Dreisigacker *et al.* 2004) and 4.8 alleles over 93 loci among 95 eastern U.S. soft winter wheat lines and cultivars (Bresghehello and Sorrells 2006). In contrast, Hao *et al.* (2011) reported allele number 13.1 per locus in 250 Chinese bread wheat lines; Roussel *et al.* (2004) reported allele number 14.5 and gene diversity of 0.662 among 559 French wheat accessions over 42 polymorphic loci. These higher allelic numbers and gene diversity information may be attributed to the use of a high number of germplasm lines, use of landraces and diverse germplasm maintained at gene bank. The PIC values revealed significant variability among different SSRs in terms of molecular polymorphism, which is in agreement to the report of Prasad *et al.* (2009). Further computing the MI facilitated unrevealing the allelic richness of SSRs within these WSHs where chromosomes 1D, 3D and 6D possessed greater allelic richness and had slightly different (non-significant) values for MI as compared to other four D-genome chromosomes. This indicated the ability to detect marker specific polymorphisms of these chromosomes which were higher as compared to 2D, 4D, 5D and 7D allowing the inference that more genetic diversity resided within chromosomes 1D, 3D and 6D.

The chromosome specific diversity analysis (Table 3) demonstrated that a higher number of alleles may be present for traits controlled by genes within these chromosomes with some being novel that could be harnessed by efficient molecular technologies and stringent phenotypic assessment. Further, the WSHs within the six clusters identified in Fig. 1 aided in analyzing diversity trends within and between these clusters by AMOVA procedures. Upon combining the

variability explained amongst and between clusters (22.45%), it became apparent that genetic variability in WSHs was higher than that reported in US hard red winter wheats (Prasad *et al.* 2009) and European winter wheat collection (Karsai *et al.* 2012). Similar trends were observed for the durum groups where genetic diversity within and between durum groups accounted for 23.15% of the total variability. Pair-wise Wright's fixation index further resolved the relationship between each cluster (Table 6) and the durum group (Table 7) in quantitative terms. The greater presence of significant differences between the cluster pairs as compared to the durum groups indicated that the diversity enhancement may be classified as true clusters as compared to durum groups. The significant *Fst* values between the pairs revealed that a real difference between clusters exists indicating a significant amount of genetic variability amongst WSHs.

Parallel analysis of phenotypic and genotypic diversity enabled to distinguish WSHs with similar genotypes having different phenotypes from those of similar phenotypes with diverse genotypes. This applied information of combining the phenotypic and molecular diversity is a strong parental selection sieve for breeding and the priority WSHs can be identified in wheat improvement programs based on multiple or specific attributes of interest. Thus, wheat breeders can use these suggested winter synthetics in targeted breeding efforts.

LD-based diversity

A number of LD-based mapping and diversity studies in wheat have been conducted at the genome or chromosome levels (Brescghello and Sorrells 2006, Chao *et al.* 2007, Somers *et al.* 2004, Horvath *et al.* 2009, Chao *et al.* 2010, Hao *et al.* 2011, Maccaferri *et al.* 2011). Nevertheless it is important to characterize germplasm for examining the extent of LD to study the genetic diversity. Overall the observed LD was low in WSHs and only 4% of the marker pairs reached the threshold of 0.2 r^2 value in the collection ($N=375$; marker pairs). This value represents that the plant mating system affects LD, and generally self-fertilization leads to a more extensive LD due to several reduced effective recombination levels (Flint-Garacia *et al.* 2003). However, several variations in the LD pattern of the germplasm reflected a complexity of the evolutionary and breeding history of wheat (Dubcovsky and Dvorak 2007). The lower values of LD observed in WSHs are in concordance of what has been previously reported by Chao *et al.* (2010) using the SNP marker system. They reported that CIMMYT wheat populations with the lowest LD among completely linked loci and the slowest rate of LD decay was possibly a consequence of an intensive use of synthetic wheat lines. Synthetic wheats and their derivatives have greatly increased genetic diversity in hexaploid wheat, particularly in D-genome (Warburton *et al.* 2006). A similar case is observed in these WSHs where unusual patterns of LD, rate of LD decay and lower pairwise r^2 values are attributed to the genomic constitution of the germplasm. It is well known that the introduction of new haplotypes from divergent populations can increase the extent of LD (Pritchard and Przeworski 2001). The comparison of the LD levels/patterns obtained in our study to the previously published results dealing with wheat and other inbreeding crops was complicated essentially due to the differences in the molecular marker platform utilized and the sample size variation amongst different studies.

Association mapping strategies to identify the genes underlying the desirable traits would benefit from the knowledge of LD patterns. In our findings, r^2 values decreased with the increase of distance among markers. Similarly population used for LD analyses had a great impact on detection of alleles and finding the association between loci. The use of population with more related breeding lines, typically those adapted to the same wheat production region, may increase the level of LD and facilitate the diagnosis of association between markers and QTL using fewer markers. When using divergent germplasm (as in our study), the evolutionary history of an allele has a profound impact on the probability of detecting marker-trait associations. Alleles that represent local adaptation and recent selection can be more readily detected using an even more sparsely

distributed set of markers. Therefore studies analyzing diverse germplasm should be based on highly dense marker populations; otherwise inadequate genome coverage may result in failure to identify critical associations. However, if the efforts are only reduced to study the genetic diversity in newly developed germplasm, a smaller number of markers can also be used to achieve the objective.

In conclusion, we reported higher genetic variability and LD pattern in WSHs. To our knowledge, this is the first study to reveal the genetic diversity in winter synthetic hexaploids with the help of a relatively more powerful statistical tool of LD, complemented by basic molecular analysis tools (AMOVA and cluster analysis). The results will be useful for constituting the germplasm panel for association mapping studies where the presence of winter synthetics will be useful to identify new alleles for the agronomic and plant adaptive characteristics.

Conclusion and future prospects

The LD analyses better discriminated the genetic diversity at the chromosomal level and formed the basis for the association mapping studies by including the diverse wheat germplasm of winter growth habit. The enormous array of spring synthetic hexaploids is a unique reservoir for spring wheat breeding programs. As the spring habit is dominant, the filial advanced generations are easy to handle and progeny numbers are substantial to select from. Crossing such spring SHs onto winter wheats gives F1 combinations of a spring habit that upon segregation generate a low frequency of winter-type derivatives. The low frequency of segregating winter types offsets the optimum population target for selections that are desirable for efficient winter wheat breeding programs. Hence the winter synthetic hexaploids now produced and freely available have provided a more efficient diversity tool to winter wheat breeders. Should spring wheat breeders desire to utilize WSHs in their breeding efforts, crossing winter SHs onto spring wheat cultivars and then selecting spring types is a more amenable protocol as segregates from the spring/WSHs will give a greater spring filial generation progeny for selecting spring-type derivatives. It will also allow the spring wheat breeder access to the diversity of the winter durum genomes. As ours is a spring wheat location, the spring/WSH breeding strategy is a unique way of further widening the national wheat gene pool and complimenting other efforts to address the food security efforts underway conventionally.

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