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Original article

Association analysis for agronomic traits in wheat under terminal heat stress

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ABSTRACT

Terminal heat stress causes irreversible damage to wheat crop productivity. It reduces the vegetative growth and flowering period that consequently declines the efficiency to capture available stem reserves (carbohydrates) in grains. Markers associated with thermotolerant traits ease in marker assisted selection (MAS) for crop improvement. It identifies the genomic regions associated with thermotolerant traits in wheat, but the scarcity of markers is the major hindrance in crop improvement. Therefore, 158 wheat genotypes were subjected to genotyping with 165 simple sequence repeat markers dispersed on three genomes (A, B and D). Allelic frequency and polymorphic information content values were highest on genome A (5.34 (14% greater than the lowest value at genome D) and 0.715 (3% greater than the lowest value at genome D)), chromosome 4 (5.40 (16% greater than the lowest value at chromosome 2) and 0.725 (5% greater than the lowest value at chromosome 6)) and marker *xgwm44* (13.0 (84% greater than the lowest value at marker *xbarc148*) and 0.916 (46% greater than the lowest value at marker *xbarc148*)). Bayesian based population structure discriminated the wheat genotypes into seven groups based on genetic similarity indicating their ancestral origin and geographical ecotype. Linkage disequilibrium pattern had highest significant ($P < 0.001$) linked loci pairs 732 on genome A at $r^2 > 0.1$ whereas, 58 on genome B at $r^2 > 0.5$. Linkage disequilibrium decay ($P < 0.01$ and $r^2 > 0.1$) had larger LD block (5–10 cM) on genome A. Highly significant MTAs ($P < 0.000061$) under heat stress conditions were identified for flag leaf area (*xwmc336*), spikelet per spike (*xwmc553*), grains per spike (*cxfa2147*, *xwmc418* and *xwmc121*), biomass (*xbarc7*) and grain yield (*xcfa2147* and *xwmc671*). The identified markers in this study could facilitate in MAS and gene pyramiding against heat stress in wheat.

1. Introduction

Wheat is an important staple food crop, grown in a wide range of climatic and soil conditions. It is a source of energy and provides 70–75% calories and 8–15% proteins in a daily diet (Shewry and Hey, 2015). It is not only consumed as human diet, but a large portion of wheat is used to make flour, semolina and raw material for many bakery products. Pakistan is an agriculture-based country and wheat is grown as a staple food in Pakistan (Shaukat et al., 2021; Rizwan et al., 2021; Islam et al., 2021). Rising global warming increases the incidence of disease, changes in the rainfall pattern and overall temperature of the earth (Ahmed, 2020, Ahmed et al., 2020, Ahmad et al., 2019; Ahmed et al., 2019; Ali et al., 2013; Fatima et al.,2020, 2021; Ahmed, 2017; van Ogtrop et al., 2014; Ahmed et al., 2014). It has been reported that mean global temperatures have risen to record 1.2 ℃ higher than previous century (Voosen, 2021). Similarly, work of Schneider et al. (2007) predicted that in 2100 it can go up to 3 ℃. Determinantal impact of climate change and drought on food and water security have been reported by Ding et al. (2021). Their results suggested that these problems could be solved by using different management options through simulation modeling (Ahmed and Hassan, 2011; Ahmed, 2012; Ahmed et al., 2013, 2014, 2016, 2018; Ahmed and Ahmad,

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2019; Ahmad et al., 2019, 2021). Protein concentartion in wheat crop was significantly affected due to climate change as reported in the work of Asseng et al. (2019). Liu et al. (2019) concluded that under 1.5 °C and 2.0 °C scenarios wheat production will change by −2.3% to 7.0% and −2.4% to 10.5% respectively. Similarly global impact of temperature of <2 °C is not evenly distributed and it will affect regional food security, food prices and trade.

Global temperature has been increased to 1.5 °C since the industrial revolution and it is predicted that increase will be 2.5–4.5 °C till 2100 year due to greenhouse gases emission (Ahmed and Stockle, 2017; Ahmed, 2017). In Pakistan, wheat planted between 15th October to 20th November and each day delay in sowing causes upto 1% yield losses (Moshatati et al., 2017; Ahmed and Farooq, 2013; Akmal et al., 2011). However, 80% of the wheat is late planted due to delay in the physiological maturity of rice and picking of cotton whereas, 20% is planted at normal time in Pakistan (Laghari et al., 2012). Due to late sowing, wheat plant faces terminal heat stress at anthesis and grain filling duration and causes significant yield losses (Aslam et al., 2017). Azmat et al. (2021) suggested early sowing of wheat under changing climate. Furthermore, Gaydon et al. (2021) concluded that improvement in the rice–wheat system water productivity is possible through optimized agronomic changes. Sowing date adjustment is commonly recommended adaptation strategy under changing climate, but it has limitations as reported by Shah et al., (2021).

Abiotic stresses prompted by different climate extreme events could affect crop growth and development. Crops genotypes which can up regulate antioxidant and stress responsive genes could withstand against abiotic stresses (Raja et al., 2020). Mineral nutrition can also be good option to alleviate heat stress in crop plants (Sarwar et al., 2019). Similarly, plant defense mechanisms under stress could be modulated through the application of growth regulators e.g. hydrogen peroxide, salicylic acid, *moringa* leaf extract and ascorbic acid (Sarwar et al., 2018). Brassinosteroids are plant steroid hormones that can induce stress tolerance in plants as reported by Kaur et al., (2018) where they investigated the affects of 28-homobrassinolide seed priming on *Brassica juncea* seedlings under heat and salinity stress. Melatonin is another good stress defender that can provide physiological protections against environmental stresses (Qi et al., 2018). Heat stress during anthesis damages the reproductive organs associated with spike fertility. High temperatures hinder the microsporogenesis and microgametogenesis which induce spore abortion subsequently reduction in grain formation (Schindfessel et al., 2021; Khan et al., 2021; Talukder et al., 2014). It also inhibits starch accumulation into grains due to granule bound starch, soluble starch and sucrose synthase enzymes activity during grain filling that consequently leads to reduction in grain size, weight and ultimately grain yield (Zahra et al., 2021; Zhao et al., 2008). Grain filling duration and grain filling rate determines grain development. Longer grain filling duration facilitates the longer time to capture available resources and improve the grain weight (Impa et al., 2021; Girousse et al., 2021; Moshatati et al., 2017).

Genetic basis of thermotolerance using genome wide association mapping is useful to improve wheat yield. Recently, mapping and identification of loci controlling thermotolerance traits utilizing microsatellite markers enhances the efficiency of crop improvement faster than conventional breeding techniques (Manjunatha et al., 2021; Mishra et al., 2021; Goel et al., 2019; Hamblin et al., 2011). Plant genomes have large numbers of microsatellite or simple sequence repeats (SSR) that may be dinucleotides, trinucleotides and so on. SSR are PCR-based and co-dominant markers. They are highly polymorphic and discriminate closely related individuals (Sharma et al., 2021; Jones et al., 2009).

Genetic mapping approaches include QTL mapping (traditional linkage mapping) and association mapping (linkage disequilibrium). Quantitative trait loci (QTLs) are the nucleotides sequence on the genome controlling the trait of interest. It requires the progenies/population developed from crosses between desirable parents which includes RILs (recombinant inbred lines), backcross progenies, F_2 population, double haploid and near isogenic lines (Kim et al., 2021a, 2021b; Bányai et al., 2021). QTL mapping have advantages viz., rare allele identification, few genetic markers requirement and no population structure effects. Whereas, disadvantages includes long period to develop populations, costly, laborious, limited detection of QTLs and few events of recombination consequently coarse mapping (Kim et al., 2021a, 2021b; Bányai et al., 2021).

Association mapping is alternative to traditional QTL mapping that determine the association between genotypic and phenotypic variation in population. Association mapping requires the presence of linkage disequilibrium that is the non-random association of alleles at different loci (Abou-Elwafa and Shehzad, 2021; Christopher et al., 2021; Kamara et al., 2021; Malik et al., 2021; Jiang et al.,2021). Linkage disequilibrium may vary due to different populations, genetic drift, mating system and recombination (Gupta et al., 2005). Understanding the linkage disequilibrium genetic pattern enhances the precision of marker trait association. Strong association between linked loci indicated the presence of linkage disequilibrium decay (Abou-Elwafa and Shehzad, 2021; Christopher et al., 2021; Kamara et al., 2021; Malik et al., 2021; Jiang et al.,2021; Stich et al., 2006). Decay of linkage disequilibrium depends on the distance in centimorgon (cM) among alleles. Christopher et al., (2021) and Rafalski, (2002) reported that when decay of linkage disequilibrium is rapid then association mapping resolution would be high.

Marker trait association helps in MAS rather than selection based on phenotypic traits. Nevertheless, limited availability of markers associated with thermotolerant traits prompted this research. Therefore, latest study was designed for the identification of linkage disequilibrium pattern in wheat genotypes to detect association between markers and agronomic traits related to terminal heat stress.

2. Materials and methods

2.1. Plant material and experimental layout

The experiment comprised of 158 wheat genotypes (Supplementary material 1) collected from NARC (National Agricultural Research Center) Islamabad, BARI (Barani Agricultural Research Institute) Chakwal, RARI (Regional Agricultural Research Institute) Bahawalpur, AARI (Ayub Agricultural Research Institute) Faisalabad, Pakistan and CIMMYT (International Maize and Wheat Improvement Center) Mexico viz., 23rd SAWYT (23rd Semi arid wheat yield trial) and 24th SAWYT (24th Semi arid wheat yield trial). Present research work was conducted at the research farm of Pir Mehr Ali Shah Arid Agriculture University Rawalpindi (33.1172°N, 73.0109°E) Pakistan. Genotypes were sown under normal (1st week of November) and heat stress (1st week of December) conditions for three years (2016–2019) in Augmented Complete Block Design with thirteen blocks using check varieties viz., AS-2002 and Aas-11. Twenty plants were selected for phenological data recording at different stages (Zadoks et al., 1974) viz., days to heading (Zadoks scale 55), days to anthesis (Zadoks scale 64), days to maturity (Zadoks scale 88), grain filling duration (Zadoks scale 69–91). Flag leaf area was measured at anthesis stage. Morphological traits that includes plant height, tillers per plant, spike length, spikelet per spike, grains per spike, thousand grain weight, biomass and grain yield per plant (Zadoks scale 91) were recorded at maturity.

2.2. Genotyping

Deoxyribonucleic acid (DNA) was extracted according to (Randhawa et al., 2009). Briefly, two pieces of one-inch leaves from 14 days old wheat seedlings were placed in each well of plate and lyophilized for 3–4 days. Metallic beads (3 mm, V & P Scientific, USA) were put in each well and shaken in Qiagen Mixer Mill, Model MM-301 (Qiagen, USA) for 6 min at 30 Hz speed. After grinding, 750 µl hot SDS extraction buffer was added and placed on levitation machine (V&P Scientific, Inc. USA) for 90 min at 60 °C to obtain 500 µl supernatant. Equal amounts of chloroform:octanol (24:1) were added in each well and centrifuged for 30 min at 4 K rpm. Then 2/3 isopropanol was added and again centrifuged for 30 min at 4 K rpm. Solution was discarded and washed the pallet twice in 70% ethanol. Pallet was dried in air and suspended the pallet in 500 µl TE buffer (500 mM Tris and 50 mM EDTA).

2.3. Marker analysis

A set of 165 simple sequence repeats (SSR) primers were randomly selected covering three genomes and M13 tail (CACGACGTTGTAAAACGAC) was synthesized with forward tail primer at 5′ end for polymerase chain reaction with each dye (FAM, HEX, NED or PET). Then 2 µl PCR products (0.5 µl PCR product containing each dye) were diluted in 10 µl loading dye (5 µl formamide and 5 µl DNA ladder) and reaction was performed on DNA analyzer machine ABI-3730 (Scientific, 2014).

2.4. Statistical analysis

Phenotypic data was analyzed by PROC MIXED with block random and entries fixed. BLUP (Best Linear Unbiased Prediction) of mean values were estimated by using statistical software SAS (Scott and Milliken, 1993). Relative performance of recorded data was estimated for agronomic traits following Asana & Williams, (1965). Broad-sense heritability was calculated using the equation:

$$H2 = \sigma^2 G/(\sigma^2 G + (\sigma^2 e/nE)$$

where σ^2G is the genotype variance, σ^2e represents the variance of the residual and nE is the environments number.

Allele frequency and PIC (Polymorphism information content) were calculated according to (Liu and Muse, 2005). Population structure was performed on STRUCTURE with burn in length 50,000 cycles and simulation length 100,000 replications. Clusters were assumed 1–12 with 5 independent runs. Evano criteria were utilized to extract DeltaK value in software STRUCTURE HARVESTER v0.6.93 (Evanno et al., 2005). Cluster analysis was performed using Jaccords method with 1000 permutations in software DARwin 6.0 (Perrier and Flori, 2003) and utilizing Unweighted pair-group method with arithmetic mean, dendrogram was constructed by FigTree v1.3.1 (Rambaut, 2009). Principal coordinate analysis was performed using software NTSYS-pc V.2.1 (Rohlf, 2000).

Linkage disequilibrium (LD) was calculated for each paired loci among P-value < 0.001 and allelic frequency correlation (r^2) in TASSEL V4.3.1 (Bradbury et al., 2007). Extent of LD was estimated at $P < 0.01$ with $r^2 > 0.1$ according to Breseghello and Sorrells (2006) and scatter plot was developed among syntenic r^2 and genetic distance in software SPSS v16.0. Relative kinship matrix was derived from unlinked markers and Q matrix obtained from Population Structure. Mixed Linear Model (MLM) was used to identify marker trait associations at $P < 0.001$ through integration of phenotypic and marker data in TASSEL V4.3.1. For stringent threshold, Bonferroni correction was calculated at $P < 0.01$ divided by studied SSR markers that was $P < 0.00061$.

3. Results

3.1. Phenotypic variation and broad sense heritability

In current study, normal planted wheat faced 25–28 °C temperature at anthesis and 28–32 °C at grain filling that was below the threshold level. Delayed planted wheat exposed to high temperature 28–31 °C at anthesis and 32–36 °C during grain filling duration that influenced the wheat crop productivity (Fig. 1). Delayed planting exposes the wheat plant to heat stress, induces preflowering and reduction in grain filling phase. Results of phenotypic traits are presented in Table 1. Genotypes and treatments were significant for all traits whereas, years were non-significant at $P < 0.001$ (Supplementary material 2). Mean performance revealed that heat stress overall reduced heading period (20.0%), anthesis (16.3%), maturity (14.1%) and grain filling duration (21.3%). Morphological traits viz., plant height (28.4%), leaf area (51.8%), spike length (18.3%), spikelet per spike (15.8%), tillers per plant (27.4%), grains per spike (19.0%), thousand grain weight (16.0%), biomass per plant (17.7%) and grain yield per plant (34.0%) were also reduced under heat stress. Broad sense heritability ranged from 86% (thousand grain weight) to 99% (days to maturity) under normal conditions whereas 71.5% (flag leaf area) to 97.4% (days to anthesis) under heat stress conditions (Table 1).

3.2. Genetic diversity

Allelic frequency and PIC value is the measures of genetic diversity among genomes and chromosomes. The 165 SSR markers were tested on genome A, B and D (59, 57 and 49) as presented in Table 2. Genome A had highest number of alleles (315 alleles, 2–13 alleles per locus) subsequently genome D (262 alleles, 3–9 alleles per locus) and genome B (236 alleles, 3–10 alleles per locus). Allelic frequency was highest on genome A (5.34) followed by genome B (4.82) and genome D (4.60). Among chromosome, highest allelic frequency (5.40) was calculated highest on chromosome 4 and marker *xgwm44* (13 alleles) (Tables 3 and 4).

Genome A showed highest PIC value (0.715) ranged 0.221–0.916 followed by genome B (0.689) ranged 0.280–0.885 and genome D (0.695) ranged 0.399–0.859. Among chromosome, PIC value was highest on chromosome 4 with PIC value 0.725 (0.520–0.916)

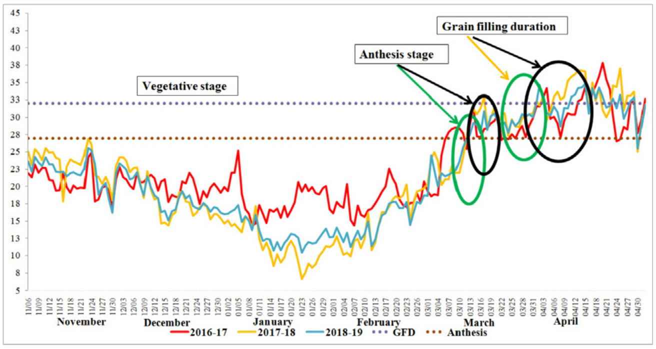

Fig. 1. Temperature data recorded at PMAS-Arid Agriculture university research farm during 2016–17, 2017–18 and 2018–19 temperature threshold level at anthesis (28 °C) and temperature threshold at grain filling duration (32 °C). Green circle represented the stage of wheat crop under normal conditions whereas black circle represented the late planted at anthesis and grain filling duration.

Table 1
Mean performance of agronomic traits under normal and heat stress conditions based on BLUPs estimates during 2016–17, 2017–18 and 2018–19.

Traits	Normal				Heat stress				RP (%)
	Mean	Range	SE	H^2	Mean	Range	SE	H^2	
Days to heading	111.8	104.1–121.3	1.184	0.96	89.4	83.1–97.5	1.037	0.94	20.0
Days to anthesis	128.3	122.1–132.8	0.528	0.97	107.4	101.9–111.7	0.512	0.97	16.3
Days to maturity	144.6	135.5–149	2.027	0.99	124.2	120.6–138.7	1.790	0.93	14.1
Grain filling duration	37.5	27.7–48.1	2.065	0.94	29.5	23.3–38.6	1.805	0.83	21.3
Plant height	87.6	79.2–97.8	1.359	0.99	62.7	53.2–71.5	2.366	0.77	28.4
Flag leaf area	33.6	22.2–47.4	0.916	0.98	16.2	11.1–21.9	2.325	0.72	51.8
Tillers per plant	6.96	5.37–9.37	0.286	0.94	5.05	3.81–7.96	0.562	0.82	27.4
Spike length	12.6	10.6–14.8	0.377	0.91	10.3	8–12.1	0.443	0.88	18.3
Spikelet per spike	21.5	18.1–24.1	0.467	93.6	18.1	14.7–21.4	0.584	0.93	15.8
Grains per spike	52.1	42.3–58.9	0.839	0.97	42.2	33.5–47.9	0.757	0.97	19.0
Thousand grain weight	36.9	34–41.4	0.902	0.86	31	26.7–34.9	0.736	0.91	16.0
Biomass per plant	54.7	46.8–63.7	1.130	0.98	45	39.1–51.4	1.200	0.93	17.7
Grain yield per plant	8.53	6.04–11	0.402	0.94	5.63	3.74–9.15	0.374	0.97	34.0

RP%: Relative performance percentage, SE: Standard error, H^2: Broad sense heritability.

Table 2
Allele frequency and PIC value on wheat genome and chromosomes using 165 SSR markers.

	Loci	Allele frequency	Mean alleles	Alleles range	PIC mean	PIC value range
A genome	59	315	5.34	2–13	0.715	0.221–0.916
B genome	49	236	4.82	3–10	0.689	0.280–0.885
D genome	57	262	4.60	3–9	0.695	0.399–0.859
Whole genome	165	813	4.92	2–13	0.699	0.221–0.916
Chromosome 1	39	196	5.02	2–10	0.696	0.450–0.879
Chromosome 2	21	95	4.52	3–7	0.695	0.399–0.839
Chromosome 3	23	105	4.56	3–9	0.712	0.516–0.872
Chromosome 4	20	108	5.40	3–13	0.725	0.520–0.916
Chromosome 5	28	129	4.61	3–9	0.678	0.221–0.829
Chromosome 6	17	91	5.35	3–8	0.691	0.280–0.846
Chromosome 7	17	89	5.24	3–8	0.723	0.475–0.859

subsequently chromosome 7 with PIC value 0.723 (0.475–0.859) and chromosome 3 with PIC value 0.712 (0.516–0.872) respectively. PIC value was highest for marker *xgwm44* (0.916), *xcfd39* (0.885) and *xgwm666* (0.879).

3.3. Population structure

Genetic similarity among wheat genotypes was assessed by STRUCTURE and distributed 158 genotypes into seven different groups based on maximum likelihood and DeltaK = 7 (Supplementary material 3). Wheat accessions were assigned in seven groups viz., G1 (38 accessions), G2 (19 accessions), G3 (12 accessions), G4 (8 accessions), G5 (27 accessions), G6 (19 accessions) and G7 (35 accessions) using membership probability (0.60) as displayed in Fig. 2. Pedigree record of diverse wheat genotypes collected from different provinces of Pakistan and genotypes from CIMMYT indicated the unexpected results develop by Population structure.

Cluster analysis was performed to compare the pattern of wheat accessions with assignments of population structure. Wheat genotypes were distributed in seven clusters viz., cluster 1 (45 accessions), cluster 2 (21 accessions), cluster 3 (19 accessions), cluster 4 (25 accessions), cluster 5 (14 accessions), cluster 6 (18) and cluster 7 (16 accessions) based on genetic dissimilarity (Fig. 3).

Principal coordinate analysis is an alternate approach to Bayesian approach. First three principal components (PCs) explained 20.9% of the total variation in which PC1 retained 10.7%, PC2 6.10% and PC3 4.10 variation (Fig. 4). It also divided the 158 wheat accessions into seven groups. These results were consistence with genotypes assignment generated by cluster analysis and population structure.

3.4. Linkage disequilibrium and linkage disequilibrium decay

Simple sequence repeats markers were used to determine the linkage disequilibrium pattern at whole genome as well as individual genome level. In this study, 165 loci identified 17,834 linked loci pairs and 84,086 unlinked loci pairs on whole genome (Table 5). Highly significant loci (P < 0.001) were calculated 1825 linked loci and 7505 unlinked loci in which 1376 linked locus pairs and 5703 unlinked locus pairs were $r^2 > 0.1$ respectively.

Genome A had 9538 linked locus pairs and 37,455 unlinked locus pairs. Highly significant locus pairs (P < 0.001 with $r^2 > 0.1$) identified 732 linked locus pairs and 2277 unlinked locus pairs. In genome B, 3928 linked locus pairs and 20,670 unlinked locus pairs were identified. Highly significant loci (P < 0.001 at $r^2 > 0.1$) were 455 linked and 1621 unlinked locus pairs. Genome D had 4368 linked locus pairs and 25,961 unlinked locus pairs. Highly significant loci (P < 0.001 with $r^2 > 0.1$) were 189 linked loci and 1805 unlinked loci were $r^2 > 0.1$. However, linkage disequilibrium decay at genome A had larger LD block within 5–10 cM genetic distance at P < 0.01 with $r^2 > 0.1$ that was larger than B (<5 cM) and D (<5 cM) genomes (Fig. 5).

3.5. Marker trait association

Marker trait associations (MTAs) identified the regions in wheat genome associated with phenoytpic traits. Stable MTAs were observed at P < 0.001 under both normal and heat stress conditions (Table 6). Days to heading was significantly associated with markers *xwmc243* (2B) and *xwmc737* (5B) whereas, days to anthesis with *xcfd219* (5B) under normal conditions. Grain filling duration

Table 3
SSR markers on chromosome with allele, base pair range and PIC.

Markers	Chrom	Allele	SRBP	PIC	Markers	Chrom	Allele	SRBP	PIC
xgwm164	1A	5	125–183	0.773	xbarc197	5A	5	138–183	0.767
xcfa2219	1A	4	226–263	0.744	xgwm154	5A	5	188–227	0.729
xgwm136	1A	10	130–276	0.834	xgwm291	5A	3	113–188	0.494
xbarc17	1A	7	135–255	0.793	xbarc141	5A	3	268–284	0.640
xbarc119	1A	7	112–177	0.773	xcfa2155	5A	5	242–351	0.221
xwmc183	1A	4	183–211	0.692	xbarc3	6A	7	153–215	0.846
xwmc716	1A	7	126–192	0.693	xwmc553	6A	8	96–182	0.773
xgwm666	1A	6	97–156	0.879	xwmc201	6A	6	152–217	0.776
xbarc148	1A	2	205–217	0.496	xgwm334	6A	3	289–336	0.516
xwmc312	1A	7	132–277	0.790	xcfd30	6A	6	218–292	0.803
xbarc83	1A	6	235–267	0.735	xgwm169	6A	4	189–197	0.672
xwmc24	1A	5	127–143	0.785	xbarc171	6A	6	223–238	0.718
xwmc304	1A	4	122–146	0.638	xbarc219	7A	3	202–226	0.475
xwmc278	1A	3	146–177	0.666	xbarc49	7A	6	153–197	0.801
xgwm135	1A	9	117–198	0.637	xgwm332	7A	7	208–246	0.824
xgwm357	1A	3	122–149	0.597	xgwm63	7A	6	104–191	0.775
xwmc469	1A	8	115–195	0.841	xgwm233	7A	4	163–197	0.715
xcfd15	1A	5	123–187	0.701	xcfa2257	7A	7	129–241	0.752
xwmc336	1A	4	95–128	0.653	xgwm11	1B	6	122–173	0.665
xcfa2129	1A	6	126–171	0.759	xgwm18	1B	4	113–147	0.575
xwmc150	2A	7	169–223	0.839	xbarc8	1B	3	171–195	0.614
xgwm47	2A	4	241–275	0.628	xcfa2147	1B	4	165–197	0.735
xgwm210	2A	6	176–204	0.806	xgwm403	1B	4	134–142	0.680
xbarc231	2A	3	268–282	0.630	xwmc367	1B	4	144–175	0.689
xgwm515	2A	4	123–149	0.713	xgwm153	1B	3	178–182	0.600
xwmc261	2A	6	209–263	0.771	xwmc52	1B	3	229–233	0.484
xwmc532	3A	5	104–168	0.783	xwmc243	2B	4	239–269	0.740
xcfa2076	3A	3	269–292	0.616	xgwm55	2B	6	122–146	0.785
xbarc113	3A	4	116–163	0.667	xbarc35	2B	3	339–345	0.444
xwmc11	3A	4	162–196	0.690	xbarc92	2B	4	193–253	0.734
xbarc12	3A	6	158–206	0.780	xgwm429	2B	6	168–221	0.720
xbarc78	4A	5	137–163	0.713	xwmc175	2B	7	243–340	0.734
xgwm494	4A	6	168–224	0.796	xbarc349	2B	4	93–105	0.650
xwmc89	4A	4	213–247	0.707	xbarc7	2B	5	143–189	0.732
xbarc170	4A	4	151–175	0.710	xbarc75	3B	3	106–110	0.522
xcfa2256	4A	3	211–229	0.649	xwmc27	3B	4	271–309	0.652
xgwm44	4A	13	126–197	0.916	xwmc326	3B	4	110–167	0.683
xgwm601	4A	8	131–148	0.805	xbarc102	3B	5	147–192	0.755
xgwm160	4A	4	97–129	0.810	xwmc418	3B	4	133–177	0.699
xwmc110	5A	7	243–304	0.829	xbarc68	3B	9	211–293	0.851
xbarc186	5A	3	291–334	0.535	xgwm131	3B	4	108–132	0.872

Chr: Chromosome location, BP: Base pair, PIC: Polymorphic information content, SRBP: Size range in base pair.

was linked with *xcfd219* (5B) and *xwmc153* (3A) whereas, spikelet per spike with *xwmc304* (1A). Marker *xgwm335* (5B) was associated with plant height and *xcfd42* (6D) with thousand grain weight. Grain yield was significantly linked with two markers viz., *xwmc28* (4A) and *xdgm153* (3A) under normal conditions.

Days to anthesis was significantly linked to three markers viz., *xwmc312* (1A), *xcfa2256* (4A) and *xwmc243* (2B) whereas, days to maturity with *xwmc473* (4D). Flag leaf area was associated with markers *xmwc336* (1A) and *xcfa2256* (4A). Marker *xgwm335* (5B) was significantly linked with plant height, *xwmc278* (1A) with spike length and *xgdm153* (5D) with thousand grain weight. Spikelet per spike was associated with three markers viz., *xwmc553* (6A), *xbarc142* (5A) and *xbarc54* (6D). Grains per spike with nine markers viz., *xwmc11* (1A), *xcfa2155* (5A), *xcfa2147* (1A), *xwmc27* (3B), *xwmc418* (3B), *xgdm111* (1D), *xgdm72* (3D), *xcfd76* (6D) and *xwmc121* (7D). Biomass per plant was stably associated with markers *xcfa2129* (1A), *xbarc148* (1A), *xbarc7* (2B), *xgwm335* (5B) and *xgdm153* (5D) under heat stress conditions. Grain yield was signifantly associated with ten markers viz., *xwmc11* (1A), *xcfa2256* (4A), *xbarc197* (5A), *xbarc8* (1B), *xgwm18* (1B), *xcfa2147* (1B), *xwmc737* (6B), *xgdm72* (3D), *xcfd76* (6D) and *xwmc671* (7D) under heat stress conditions.

Bonferroni correction for stringent threshold identified highly significant MTAs at P < 0.000061 under heat stress conditions. Marker *xwmc336* (*1A*) associated with flag leaf area whereas, *xwmc553* (6A) with spikelet per spike. Markers *cxfa2147* (1A), *xwmc121* (7D) and *xwmc418* (3B) were linked to grains per spike. Marker *xbarc7* (2B) was tightly linked with biomass whereas, markers *xcfa2147* (1A) and *xwmc671* (7D) with grain yield under heat stress conditions.

4. Discussion

4.1. Phenotypic traits

Terminal heat stress is an important concern in subtropical, tropical, semi-arid and arid regions of the world that reduces the wheat productivity drastically. Optimum temperature for normal wheat growth at heading is 15–20 °C, anthesis 22–25 °C and grain filling duration is 25–28 °C (Asseng et al., 2015; Tack et al., 2015). But temperature above the threshold level at heading (20 ± 1.6 °C), anthesis (26 + 1.01 °C) and grain filling duration (30 + 2.13 °C) negatively affects the wheat crop productivity (Khan et al., 2020). In current study, delayed planted crop faced high temperature at anthesis (28–31 °C) and grain filling duration (32–36 °C) that was 3–4 °C above the threshold level and caused reduction in yield related attributes. Delayed planted genotypes compensate this temperature by reducing their vegetative and reproductive growth phases with reduction in the efficiency of physiological process and

Table 4
SSR markers on chromosome arm with allele base pair range and PIC.

Markers	Chrom	Allele	SRBP	PIC	Markers	Chrom	Allele	SRBP	PIC
xbarc147	3B	4	177–227	0.601	xbarc168	2D	3	172–178	0.588
xwmc527	3B	4	265–315	0.690	xcfd116	2D	4	241–266	0.719
xgwm149	4B	3	149–163	0.638	xcfd9	3D	5	218–265	0.762
xbarc193	4B	7	275–309	0.846	xbarc71	3D	5	175–219	0.758
xcfd39	4B	10	157–213	0.885	xgdm72	3D	6	241–334	0.775
xwmc47	4B	4	142–160	0.563	xcfd55	3D	4	209–291	0.716
xbarc163	4B	4	193–229	0.778	xcfd219	3D	6	118–305	0.819
xwmc75	5B	3	178–225	0.533	xbarc42	3D	3	159–184	0.516
xgdm146	5B	3	172–199	0.613	xcfd70	3D	4	195–209	0.732
xgwm335	5B	7	203–225	0.809	xgwm52	3D	4	142–148	0.684
xgwm234	5B	7	227–247	0.767	xgwm161	3D	5	162–206	0.762
xcfd60	5B	3	171–175	0.662	xgwm194	4D	4	117–143	0.644
xwmc160	5B	6	98–197	0.771	xgdm125	4D	9	188–265	0.825
xwmc28	5B	7	114–168	0.823	xcfd54	4D	5	143–262	0.754
xgwm408	5B	9	146–194	0.807	xwmc473	4D	3	127–173	0.520
xbarc28	5B	5	93–126	0.667	xcfd106	4D	3	162–206	0.547
xbarc156	5B	3	143–179	0.655	xwmc331	4D	5	173–226	0.700
xwmc73	5B	6	106–187	0.762	xwmc285	4D	4	162–195	0.692
xwmc737	6B	5	277–353	0.673	xcfd7	5D	5	143–262	0.754
xgwm191	6B	6	122–144	0.659	xgdm153	5D	5	183–227	0.760
xgwm193	6B	3	156–176	0.280	xgwm190	5D	3	112–187	0.547
xgwm146	7B	4	163–192	0.744	xcfd67	5D	4	201–237	0.631
xwmc76	7B	3	252–276	0.635	xbarc130	5D	5	252–280	0.607
xwmc323	7B	6	152–182	0.745	xgdm116	5D	3	153–178	0.667
xbarc32	7B	5	167–215	0.755	xgwm271	5D	4	163–179	0.749
xgwm46	7B	6	142–174	0.806	xcfd29	5D	4	177–189	0.750
xbarc169	1D	3	112–128	0.543	xcfd40	5D	3	132–158	0.821
xcfd83	1D	5	151–189	0.787	xwmc233	5D	3	122–146	0.616
xgdm33	1D	6	126–173	0.705	xbarc54	6D	4	148–181	0.695
xcfd92	1D	5	110–219	0.720	xcfd76	6D	6	224–276	0.779
xcfd63	1D	4	134–206	0.688	xgdm108	6D	7	107–136	0.830
xgwm642	1D	3	147–206	0.450	xbarc96	6D	3	163–188	0.543
xgdm111	1D	5	232–277	0.724	xcfd135	6D	5	163–208	0.715
xwmc153	1D	5	145–183	0.639	xcfd49	6D	5	172–236	0.782
xcfd48	1D	6	245–283	0.826	xcfd42	6D	7	179–235	0.688
xwmc216	1D	4	157–196	0.500	xbarc184	7D	6	132–192	0.801
xcfd72	1D	7	242–254	0.847	xwmc463	7D	4	127–169	0.694
xbarc142	2D	3	110–147	0.785	xwmc121	7D	6	265–322	0.793
xgwm157	2D	3	173–194	0.663	xcfd68	7D	3	208–232	0.502
xbarc11	2D	3	302–308	0.399	xbarc105	7D	8	128–161	0.859
xcfd168	2D	6	109–156	0.766	xwmc671	7D	5	96–147	0.763
xcfd53	2D	4	190–279	0.743					

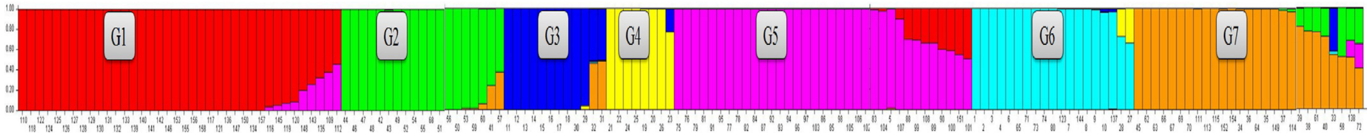

Fig. 2. Population Structure of wheat genotypes analyzed by 165 SSR markers representing 7 clusters based on their genetic similarity.

metabolic activities (Impa et al., 2021; Chaudhry and Sidhu, 2021; Zhu et al., 2021).

High temperature of 4–5 °C above the optimum temperature at booting, heading, anthesis and post anthesis period declines the crop productivity due to fast completion of different growth phases (Nawaz et al., 2013). Photoperiodic (*PPD-D1, PPD-A1)* and vernalization (*VRN1, VRN2*) responsive genes determines the plant development at volatile temperatures and limiting variuos growth phases (Zhu et al., 2021). It enforces the plant to complete its vegetative growth and enter in reproductive stage that consequently reduces the plant height and fertile tillers (Zhu et al., 2021). Heat stress also reduces the flowering period and consequently declines the efficiency to capture available stem reserves (Talukder et al., 2014; Zhu et al., 2021). Furthermore, heat stress at grain filling inactivates the metabolism of starch synthesis and accumulation into grains that determines the grains weight (Khadka et al., 2020a,b; Zhao et al., 2015; Zhu et al., 2021).

4.2. Genetic diversity

Simple sequence repeats markers distinguish the closely related genotypes due to existence of hyper variable regions in genome (Pidigam et al., 2021; Kim et al., 2021a, 2021b). In current study, 165 SSR markers were utilized to observe genetic diversity among 158 wheat genotypes. Overall, 813 alleles (average 4.92 alleles per locus) with PIC value 0.699 (0.221–0.916) on whole-wheat genome that was higher than previous studies utilizing SSR markers. Allelic frequency and PIC value were 5.5 and 0.600 (Zarei Abbasabad et al., 2016), 4.3 and 0.650 (Hao et al., 2011), 4.8 and 0.610 (Dodig et al., 2010) and 4.6 and 0.653 (Liu et al., 2010) were reported respectively. It might be due to diverse panel and polymorphic markers used in current study (Abou-Elwafa and Shehzad, 2021; Sharma et al., 2021). Allelic frequency and PIC value on each locus determine the genetic diversity on each genome or chromosome. Higher the average allelic frequency and PIC value indicates the higher

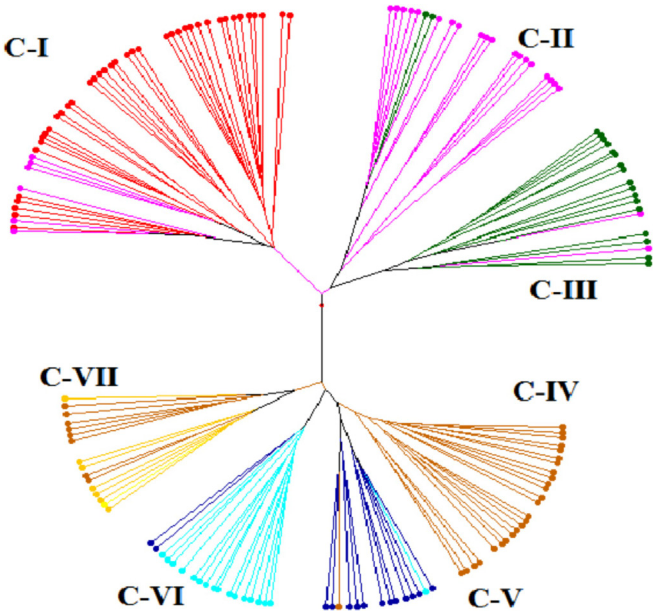

Fig. 3. Neighbor joining cluster analysis based on genetic dissimilarity using Jaccords method with 1000 permutations. Different color lines represent the each cluster viz., C-I, C-II, C-III, C-IV, C-V, C-VI and C-VII associated with population structure.

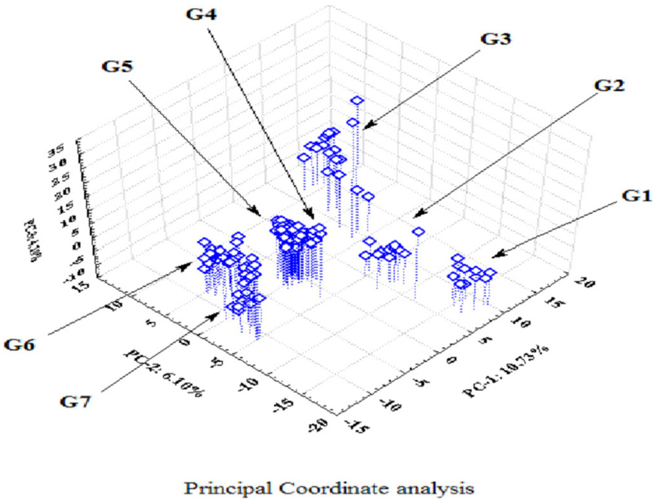

Fig. 4. Principal coordinate analysis using SSR markers. Labels G1, G2, G3, G4, G5, G6 and G7 correspond to subpopulations/groups of wheat accessions.

genetic diversity. Current study revealed the allelic frequency and PIC values on individual genome as well as each chromosome of wheat (Sharma et al., 2021; Seetharam et al., 2021). However, allele frequency and PIC values were observed from lowest to highest D < B < A genome that indicated lowest genetic diversity on D and B genome of wheat as compared to A genome in Pakistani post-green revolution varieties and CIMMYT lines

4.3. Population structure

Population structure assigned 158 wheat accessions of Pakistani post-green revolution varieties into seven subgroups. Three methods viz., population structure, cluster analysis and principal coordinate analysis consistently led this grouping. Consistency of grouping using these three methods has also been reported earlier (Ya et al., 2017; Tascioglu et al., 2016). Population structure differentiation is based on relatedness frequency of accessions to each group as hypothesized by STRUCTURE. UPGMA clustering distributed the wheat accession based on their genetic dissimilarity (Khadka et al., 2020a,b) whereas, principal coordinate analysis was based on their genetic distance. Bayesian approach population structure determined seven groups. Prior, two main groups were expected based on their origin viz., Pakistani varieties and CIMMYT lines but there was greater genetic diversity among wheat accessions which led to seven groups. The unexpected population structure (7 groups not 2 groups) results may be due to numerous factors viz., selection of germplasm by breeders, geographical origin and age of varieties that influence the structure of wheat accessions. Cluster analysis and principal coordinate analysis also validate the results by distributing wheat genotypes into seven groups.

The main source of wheat germplasm in Pakistan was brought from CIMMYT starting from 1965 when Semi dawarf Mexi-pak was released for general cultivation (Khan and Tsunoda, 1970). Mostly accessions were introduced from CIMMYT and crossed with local cultivars to develop new varieties in Pakistan. It might be due to sharing of their common parentage viz., PASTOR, SOKOLL, KAUZ and WBLL among CIMMYT lines and Pakistani accessions. For example, Pakistani variety AAS-11, Dharabi-11 and Ihasan-16 sharing common parentage PASTOR with CIMMYT lines viz., G-302, G-305, G-313, G-317, G-318, G-321, G-322, G-324, G-327 and so on (supplementary material 1). These results indicated that Pakistani genotypes used in the present study sharing the common ancestor due to their genetic similarity within the populations of a group. Additionally, population genetic structure is perquisite for marker trait association to reduce false associations. Therefore, mixed linear model (MLM) was used to remove these false associations in which Kinship matrix (difference in genetic relatedness) and

Table 5
Linkage disequilibrium pattern on wheat genomes at $P < 0.001$.

Genome	Linked locus pair				
	Observed	$P < 0.001$ (%)	$r^2 > 0.1$(%)	$r^2 > 0.2$(%)	$r^2 > 0.5$(%)
Genome A	9538	1028	732	236	21
Genome B	3928	558	455	243	58
Genome D	4368	239	189	109	31
Whole genome	17,834	1825	1376	588	110
Genome	Unlinked locus pair				
	Observed	$P < 0.001$ (%)	$r^2 > 0.1$(%)	$r^2 > 0.2$(%)	$r^2 > 0.5$(%)
Genome A	37,455	3273	2277	736	62
Genome B	20,670	2001	1621	981	322
Genome D	25,961	2231	1805	1097	390
Whole genome	84,086	7505	5703	2814	774

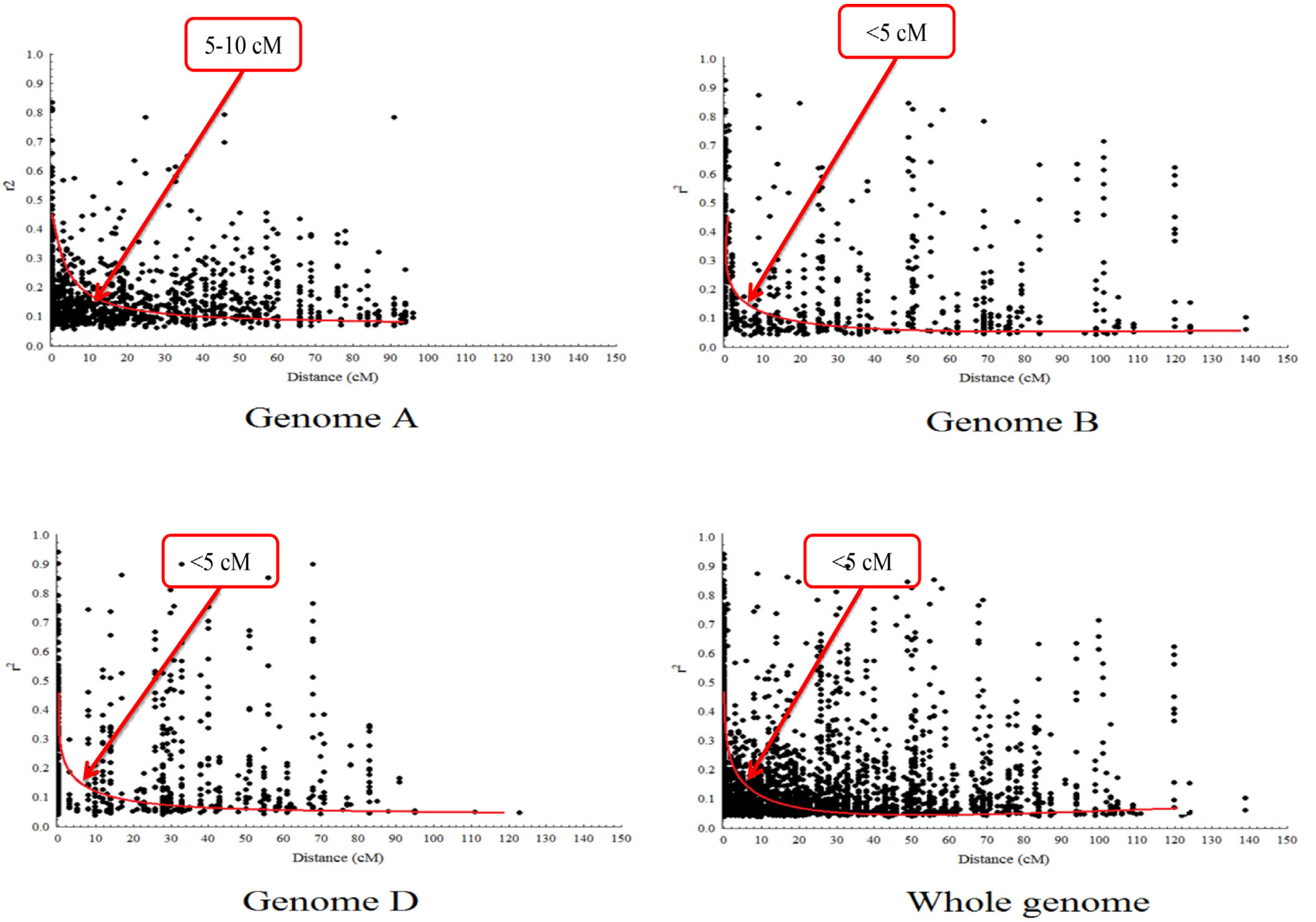

Fig. 5. Scatter plot representing linkage disequilibrium decay using r^2 values significance at $P < 0.01$ with genetic distance in cM for wheat genome A, B, D and whole genome.

population structure (Q matrix) were included in previous studies (Yu and Buckler, 2006; Zhao et al., 2007).

4.4. Linkage disequilibrium and linkage disequilibrium decay

Linkage disequilibrium is a non-random association of alleles at different loci on the chromosomes that determine the resolution of association mapping. Current study indicated the presence of linkage disequilibrium on genome and chromosome level at $P < 0.001$ with $r^2 > 0.1$ that was the prerequisite for association mapping. Higher level of linkage disequilibrium is expected in wheat than other crops due to high rate of inbreeding. Flint-Garcia et al. (2003) suggested higher level of linkage disequilibrium in wheat than cross pollinated crops viz., sorghum and maize. Thus it was necessary to find the linkage disequilibrium blocks that does not split into smaller blocks. It also determines the number of markers required for association mapping (Dadshani et al., 2021).

Linkage disequilibrium decay indicates the recombination rate which determines the association mapping precision (El-Esawi et al., 2018). Various factors viz., genetic drift, population size, admixtures, selection, mutation and non-random mating leads to variation in LD pattern (Vos et al., 2017). Decay of linkage disequilibrium depends on the distance in centimorgan (cM) among alleles. If linkage disequilibrium decays within the shorter genetic distance then association mapping resolution would be high and vice versa (Dadshani et al., 2021; Breseghello and Sorrells, 2006). In current study, linkage disequilibrium decay was larger on genome A (5–10 cM) that was larger than recent study reported by Sukumaran et al. (2015) who found decay of linkage disequilibrium within 5 cM on D genome whereas, about 2 cM on A and B genome with highly significant paired loci ($P < 0.01$) and $r^2 = 0.02$ in wheat whereas, Zarei Abbasabad et al. (2016) demonstrated LD decay up to 40–60 cM at lower $r^2 = 0.05$ in Iranian wheat varieties. This might be due to higher genetic diversity in Pakistani germplasm used in this study.

4.5. Marker trait association

Marker trait associaion application is promising approach in plant breeding to deal with the limitations faced in linkage mapping (Sharma et al., 2021; Seetharam et al., 2021; Kraakman et al., 2004). It improves the efficiency and precision of indirect selection of thermotolerance traits in breeding programs. In current study we identified significant stable marker trait associations under normal and heat stress conditions at $P < 0.001$. But using stringent threshold level (Bonferroni correction $P < 0.000061$) we could not found any significant MTA under normal conditions whereas, 8 MTAs were observed under heat stress. MTAs for grains per spike were observed on chromosome *1A*, *7D* and *3B* in present study. MTAs for grains per spike were previosuly also reported on chromosome *1A*, *4A*, *2B*, *3B* and *5B* under heat stress conditions (Shi et al., 2017). Each significant MTA was identifiend on *1A*, *6A* and *2B* for flag leaf area, spikelet per spike and biomass per plant respectively whereas, only two MTAs were identified on *1A* and *7D* under heat stress conditions. MTAs for different agronomic traits on *3A*, *4A*, *6A*, *1B*, *2B*, *2D* and *6D* were previosuly reported normal condi-

Table 6
Marker trait associations for agronomic traits under normal and heat stress conditions.

Trait	Marker	Ch	cM	P	R^2
Normal conditions					
DAH	xcfd219.2	5B	80.2	0.000327	0.0722
DAA	xwmc243.2	2B	27.0	0.000821	0.0511
DAA	xwmc737.2	6B	54.0	0.000316	0.0466
GFD	xcfd219.2	5B	80.2	0.000684	0.0655
PH	xgwm335.3	5B	78.0	0.000615	0.0737
FLA	xwmc737.4	6B	54.0	0.000747	0.0467
SPS	xwmc304.3	1A	58.0	0.000746	0.0895
GPS	xwmc153.3	3A	4.5	0.000568	0.0926
TGW	xcfd42.1	6D	39.0	0.000538	0.0982
GY	xwmc28.5	5B	144	0.000122	0.1057
GY	xgdm153.5	5D	63	0.000897	0.0812
Heat stress conditions					
DAA	xwmc312.5	1A	76.0	0.00032	0.0497
DAA	xcfa2256.3	4A	38.5	0.00079	0.0430
DAA	xwmc243.2	2B	27.0	0.00046	0.0592
DM	xwmc473.1	4D	23.0	0.00046	0.0842
PH	xgwm335.3	5B	78.0	0.00031	0.0791
FLA	xwmc336.1	1A	34.0	0.00007	0.1158
FLA	xcfa2256.2	4A	38.5	0.00097	0.0640
SL	xwmc278.2	1A	62.0	0.00096	0.0913
SPS	xwmc553.6	6A	46.0	0.00004	0.1095
SPS	xbarc142.4	5A	48.0	0.00076	0.0921
SPS	xbarc54.3	6D	42.0	0.00024	0.1079
GPS	xwmc11.2	3A	13.0	0.00052	0.0779
GPS	xcfa2155.2	5A	153.0	0.00090	0.0562
GPS	xcfa2147.4	1B	108.8	0.00001	0.1271
GPS	xwmc27.1	3B	59.0	0.00090	0.0562
GPS	xwmc418.2	3B	73.0	0.00000	0.1391
GPS	xgdm111.1	1D	7.0	0.00090	0.0562
GPS	xgdm72.3	3D	34.0	0.00029	0.0845
GPS	xcfd76.3	6D	68.0	0.00026	0.0687
GPS	xwmc121.3	7D	83.0	0.00009	0.0791
TGW	xgdm153.5	5D	63.0	0.00044	0.0982
BM	xcfa2129.3	1A	79.0	0.00053	0.0745
BM	xbarc148.2	1A	59.0	0.00067	0.0716
BM	xbarc7.3	2B	51.0	0.00002	0.1421
BM	xgwm335.3	5B	78.0	0.00074	0.0704
BM	xgdm153.5	5D	63.0	0.00078	0.0892
GY	xwmc11.2	1A	13.0	0.00018	0.1007
GY	xcfa2256.2	4A	38.5	0.00086	0.0636
GY	xbarc197.1	5A	117.0	0.00029	0.0947
GY	xbarc8.1	1B	25.0	0.00091	0.0629
GY	xgwm18.1	1B	33.0	0.00060	0.0676
GY	xcfa2147.4	1B	108.8	0.00004	0.1201
GY	xwmc737.2	6B	54.0	0.00035	0.0736
GY	xgdm72.3	3D	34.0	0.00028	0.0951
GY	xcfd76.1	6D	68.0	0.00060	0.0676
GY	xwmc671.1	7D	111.0	0.00009	0.1095

Chr: Chromosome, P: Probability, R^2: Correlation coefficient, DAH: Days to heading, DAA; Days to anthesis, DM: Days to maturity, GFD: Grain filling duration, PH: Plant height, FLA: Flag leaf area, SL: Spike length, SPS: Spikelets per spike, TP: Tillers per plant, TGW: Thousand grain weight, GPS: Grains per spike, BM: Biomass per plant, GY: Grain yield per plant.

tions (Sharma et al., 2021; Seetharam et al., 2021; Gupta et al., 2015; Ain et al., 2015; Zhao et al., 2015) that was different from markers identified and grwoing conditions in current study. In summary, few markers associated with traits were identified previously on same chromosomes under normal conditions, although heat stress conditions investigated in present study was different from previous studies. It is suggested that significant markers associated with traits under heat stress conditions would be useful in MAS that facilitates the indirect selection of traits rather than selection based on phenotype in further wheat breeding programs against heat stress.

5. Conclusion

Marker trait associations of agronomic traits expedite the efficiency of breeding programs for developing thermotolerant cultivars. Genetic rich regions were identified highest on genome A (A > B > D) in studied wheat genotypes that facilities in targeting this genome to identify more loci related to desired traits. This will also help to enhance the power of genome studies and identification of candidate genes in wheat. Population structure and LD pattern provides the useful information for marker trait association. Population structure, cluster analysis and principal coordinate analysis distributed wheat accessions into seven distinct groups representing genetically diverse germplasm. LD pattern was highly significant ($P < 0.001$) with high $r^2 > 0.1$ value suggested the presence of linkage disequilibrium on wheat genome and chromosomes that was the prerequisite of association mapping. LD decay (5–10 cM) on genome A suggested that this genome requires fewer number of markers to detect target loci related to desirable traits using association mapping than other genomes. Stable significant (Bonferroni correction $P < 0.000061$) marker trait associations identified under heat stress conditions facilitates the breeding program using MAS and gene pyramiding in wheat.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Authors contribution

Author MuA (Munir Ahmad), MA (Mukhtar Ahmed) and AK (Adeel Khan) develop the idea, AK conducted the experiment. MuA, MA, and AK have written the manuscript. Phenotypic data was collected in Pakistan under MuA supervision and genotypic data in USA under KSG. MuA, MA, ZA (Zahid Akram) and KSG (Kulvinder Singh Gill) reviewed and edited the manuscript. MA incorporated all suggestions made by the valaublе reviewers.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.sjbs.2021.08.050.

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