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NA Delvadiya
Department of Biotechnology, Junagadh Agriculture University, Junagadh, Gujarat, India

DR Mehta
Department of Genetics and Plant Breeding, Junagadh Agriculture University, Junagadh, Gujarat, India

AK Nandha
Department of Biotechnology, Junagadh Agriculture University, Junagadh, Gujarat, India

Corresponding Author:
NA Delvadiya
Department of Biotechnology, Junagadh Agriculture University, Junagadh, Gujarat, India

Construction of linkage mapping and identification of quantitative trait loci (QTL) for grain size and related traits in bread wheat (*Triticum aestivum* L.)

NA Delvadiya, DR Mehta and AK Nandha

Abstract
Grain size is an important agronomic trait that influences the grain yield and milling quality of wheat. In this paper, the experimental material comprised of P_1, P_2, F_1, F_2 and $F_{2:3}$ generations of wheat cross GW-11 X GW-322 for grain size and related traits respectively to fulfill the objective of linkage and QTL mapping in bread wheat. Out of 200 SSR markers screened for parental polymorphism for grain size and related traits, about 23% of SSR markers showed good polymorphism between two parental lines. Out of 46 tests for calculated chi-square, 42 test markers do not deviate significantly from expected ratios revealing that observed data are in agreement with expected ratio of 1:2:1. The linkage map was constructed using software IciMapping v.4.1 and Recombination frequencies were converted into map distance using Kosambi's mapping function. The markers were grouped with minimum logarithm of the odds (LOD) of 3.0 with walking speed was set at 1.0 cM. Seven linkage groups with a total map length of 77.31 cM were constructed using data from 46 SSR marker loci for 74 F_2 plants which ranged from minimum of 2.74 cM (LG2) to maximum of 26.89 cM (LG3). Genotypic data of F_2 and phenotypic data of on 74 $F_{2:3}$ lines were analyzed for identification of the main effect QTLs using the software ICIM-ADD mapping in QTL IciMappingV4.1. A linkage map of grain size and related traits output data file was used for the construction of QTL mapping. A total six QTL had been identified for grain size and related traits, one each for 100-grain weight; number of grain per the main spike (NGPMS); grain yield per plant (GYPP); number of effective tillers per plant (NETPP) and two QTLs for grain weight per the main spike (GWPMS) and could be used for marker assisted selection after validation.

Keywords: Linkage mapping, QTL mapping, SSR marker, Bread wheat

Introduction
Wheat (*Triticum* spp.), originated in the Fertile Crescent region of the Near East, belongs to the Poaceae family and is one of the most important cereal crops in the world. Most wheat cultivated today can be classified into two types, durum and bread wheat. Durum wheat (*Triticum turgidum* L. var. *durum*, 2n = 4x = 28, AABB) is a tetraploid hard wheat with an amber color, which is coarsely ground into semolina and used to make pasta and other semolina products (Feldman *et al.*, 1995; Kihara, 1944; McFadden and Sears, 1946) [10, 15, 17]. The bread wheat (hexaploid with chromosome number 2n=6x=42) is cultivated in all the wheat growing areas of the world, Wheat is the second most important staple and most widely cultivated food crop next to rice, consumed by nearly 35% of the world population and providing 20% of the total food calories. It is known for its remarkable adoption to a wide range of environments and its role in world economy. Globally, demand for wheat by the year 2020 is forecast at around 950 million tonnes per year. This target will be achieved only, if global wheat production is increased by 2.5% per annum (Clayton *et al*., 2006) [5].
Present-day Indian wheat have optimum characters needed for chapati making, but are not ideal for industrial food products like biscuits, bread and noodles. To have a good bread, characters like high loaf volume, uniform and small grains in the crumb, smooth crust and hardness of grain should be present optimally in a wheat variety. A very good understanding of, and ability to manipulate oligogenic and polygenic traits is offered to plant breeders by recent advances in genetic marker technology (Young, 1999) [32]. Use of molecular techniques for detecting differences in the DNA of individual plants has many applications for crop improvement. DNA-based molecular markers are the most powerful diagnostic tools to detect DNA polymorphism both at the level of specific loci and at the whole genome level. Simple sequence repeats (SSRs) or microsatellites markers consist of di-, tri-,or tetra-nucleotide repeats and DNA sequences flanking the repeats are used as priming sites in PCR reactions.

The amplified product contains variable number of repeats depending upon the species and generates length polymorphism. Due to the high variability of number of repeats within a microsatellite even among the members of the same species, microsatellites tend to detect a high degree of polymorphism. SSRs are typically co-dominant and have high locus specificity. Hence, they have been used extensively to develop genetic maps in wheat (Roder *et al.* 1998; Somers *et al.* 2004) [20, 23].

Grain size and shape, which are associated with milling quality (Breseghello and Sorrells 2006; Evers *et al.*, 1990) [3, 9], are two of the main targets for wheat breeding. Grain size is mainly characterized by grain weight and area, whereas grain shape means a relative proportion of the main growth axes of the grain (Breseghello and Sorrells, 2007; Gegas *et al.*, 2010) [2, 12]. Grain shape is generally estimated by length, width, vertical perimeter, sphericity and horizontal axes proportion (Breseghello and Sorrells, 2007) [2]. Many studies have identified quantitative trait loci (QTLs) for grain size and shape in common wheat cultivars and the QTLs have been assigned to various chromosomes (Breseghello and Sorrells 2006; 2007; Gegas *et al.* 2010; Sun *et al.* 2009; Tsilo *et al.* 2010; Williams *et al.*, 2012) [3, 2, 12, 26, 27, 30]. In addition, significant association with one-thousand-grain weight has been observed in some wheat orthologs of rice grain size-controlling genes (Zhang *et al.*, 2012) [33].

Materials and Methods

Seed material

The experimental materials comprised two diverse parents namely GW-11, and GW-322 which were collected from Wheat Research Station, Junagadh Agricultural University, Junagadh. The parental line GW-11 has the bold grain size; parental line GW-322 has small grain size.

Raising of parents and crossing programme

The seeds of pure lines GW-11 and GW-322 for grain size was sown at Wheat Research Station JAU, Junagadh during winter 2013-14. The parental lines and F_1 hybrid seeds were sown during winter 2014-15 to obtain selfed seeds of F_2. Whole spikelet of F_1 plant was covered with white parchment paper bags to prevent any unwanted cross pollination. Along with parental lines and saved F_1, selfed seeds of F_2 were sown during winter 2015-16. All the necessary observations were recorded in parental lines, F_{1S}, F_{2S}. Plant leaf samples were also collected from every single plant for DNA extraction 20 days after sowing and genotyping was done. To obtain selfed seeds of F_3, whole spikelet of selected F_2 plants were covered with white parchment paper bags to prevent any unwanted cross-pollination. Along with parental lines, selfed seeds of F_3 were sown in two replications at Wheat Research Station, JAU, Junagadh during winter 2016-17 for $F_{2:3}$ phenotyping.

DNA extraction

Total genomic DNA extraction was carried out by CTAB method as described by Stein *et al.* (2001) with minor modifications.

Testing parental polymorphism using SSR primers

To identify SSR primer pairs detecting polymorphism between parents, initial screening of parental lines was conducted before actual genotyping of individuals in segregation F_2 mapping population. For this, DNA from GW-11 (taken as first parent i.e. P_1) and GW-322 (taken as second parent i.e. P_2) and their corresponding F_1 hybrids were subjected to PCR amplification with each of the available SSR primer pairs. A total of 200 SSR primers pairs were used to screen the parental polymorphism of the population.

Genotyping of F_2 population

Simple Sequence Repeat (SSR) which showed good scorable polymorphic pattern in parental lines was used for characterization of F_2 population. Primers required for SSR were synthesized from Merck Bioscience, Bangalore. The amplified products of SSR were analyzed on 3% agarose gel.

Construction of Linkage Map

QTL IciMapping v4.0 (Meng *et al.*, 2015) [18] was used for linkage group construction using all the polymorphic markers. Three general steps were involved in linkage map construction: Grouping, Ordering and Rippling. First of all, markers were grouped based on a Likelihood of odd ratio (LOD) of 3.0, recombination frequency of 0.3 and Window size 5cM. To include additional markers on the map, Try and move to commands were used. Finally, linkage map based on SSR marker was constructed.

QTL Mapping

Trait data from $F_{2:3}$ was averaged for each entry and sorted to correspond with the progeny order of the genotypes (marker data). The total number of progeny individuals from the cross with trait and genotype information was 74. QTL mapping was performed using the Inclusive Composite Interval Mapping Additive (ICIM-ADD) method of QTL IciMapping v4.0. A threshold LOD score 3.0 was used to confirm significant QTL. Other parameters settings for ICIM were the largest P-value for entering variables in stepwise regression of residual phenotype on marker variables with threshold of 0.001 for removing variables and 1cM walking speed along chromosome. QTL was considered to have a significant effect when LOD statistics exceeded a threshold of 3.0 (Meng *et al.*, 2015) [18].

Results and Discussion

Parental polymorphism for grain size

The parental lines P_1 (GW-11, Bold grain) and P_2 (GW-322, Small grain) were screened against 200 SSR (microsatellite) markers to identify parental polymorphic combinations. A total of 46 polymorphic SSR markers between two parental lines were used to screen the mapping population of F_2 developed for grain size. Out of 200 markers screened, approximately 23% of SSR markers showed good polymorphism between two parental lines for grain size. All the 200 SSR makers which were used in the present study were previously reported and available in the public domain. The markers consisted primary of barc (Song *et al.*, 2005) [24], cfd (Guyomarc'h *et al.*, 2002) [14], gwm (Röder *et al.*, 1995, 1998) [21, 20], wmc (Gupta *et al.*, 2002; Somers *et al.*, 2004) [13, 23] markers. In the present study, only 23% primers were found polymorphic between two parents. Very clear and scorable polymorphisms (Fig.1) were used for genotyping the 74 individual F_2 plants across 46 marker loci. The low level of polymorphism obtained from SSR markers in the present was akin to the results reported in past in self-pollinating cereal crops like rice and wheat (Chao *et al.*, 1989; Devos *et al.*, 1992) [4, 8].

Segregation of markers and their distortion

The observed segregation pattern of marker loci (SSR) for the mapping population of 74 F_2 plants were compared with the expected ratio of 1:2:1 [1 homozygote (A) from P_1: 2 heterozygote (H): 1 homozygote from P_2 (B)]. The calculated chi-square values using observed frequency of A: H: B and its expected frequency for each and every individual marker locus.

Table 1: Chi-square tests for 46 SSR markers used to discriminate 74 F_2 equivalents to P_1, P_2 and F_1

Sr. No	Marker Name	Position	HmzA	Htz	HmzB	Missing marker	Chi-Square	Pr>ChiSq	Degree of Dominace
1	xgwm264	0.00	25	22	26	1	9.2466**	0.0098	Codominant
2	xgwm232	1.38	20	27	27	0	6.7297*	0.0346	Codominant
3	xgwm458	4.80	21	26	27	0	7.5135*	0.0234	Codominant
4	xgwm497	0.00	21	35	18	0	0.4595	0.7947	Codominant
5	xgwm210	2.74	23	31	20	0	2.1892	0.3347	Codominant
6	xgwm383	0.00	23	36	15	0	1.7838	0.4099	Codominant
7	xgwm285	4.87	18	39	17	0	0.2432	0.8855	Codominant
8	xgwm3	5.55	19	38	17	0	0.1622	0.9221	Codominant
9	xgwm493	9.70	19	36	19	0	0.0541	0.9733	Codominant
10	xgwm376	11.06	20	36	18	0	0.1622	0.9221	Codominant
11	xgwm183	14.51	19	37	18	0	0.0270	0.9866	Codominant
12	xbarc105	16.55	19	36	19	0	0.0541	0.9733	Codominant
13	xgwm645	20.00	18	39	17	0	0.2432	0.8855	Codominant
14	xgwm547	22.74	18	37	19	0	0.0270	0.9866	Codominant
15	xgwm391	26.89	18	37	19	0	0.0270	0.9866	Codominant
16	xgwm149	0.00	19	35	20	0	0.0541	0.9733	Codominant
17	xgwm610	4.86	17	39	18	0	0.2432	0.8855	Codominant
18	xwmc262	0.00	20	35	19	0	0.1622	0.9221	Codominant
19	xgwm234	2.05	21	37	16	0	0.6757	0.7133	Codominant
20	xgwm190	4.79	17	41	16	0	0.8919	0.6402	Codominant
21	xgwm126	11.21	21	35	17	1	0.5616	0.7552	Codominant
22	xgwm291	12.65	19	34	18	3	0.1549	0.9255	Codominant
23	xgwm191	16.24	22	36	16	0	1.0270	0.5984	Codominant
24	xgwm613	0.00	18	34	22	0	0.9189	0.6316	Codominant
25	xgwm508	7.05	19	35	20	0	0.1622	0.9221	Codominant
26	xwmc344	9.79	18	36	20	0	0.1622	0.9221	Codominant
27	xgwm169	11.84	19	35	20	0	0.2432	0.8855	Codominant
28	xgwm437	0.00	20	34	10	0	0.4865	0.7841	Codominant
29	xgwm43	3.59	18	34	19	3	0.1549	0.9255	Codominant
30	xbarc182	5.01	19	35	20	0	0.2432	0.8855	Codominant
31	xgwm428	9.94	17	34	22	1	1.0274	0.5983	Codominant
32	xgwm106	0.00	12	35	26	1	5.4932	0.0641	Codominant
33	gpw3254	0.00	12	35	26	1	5.4932	0.0641	Codominant
34	xwmc48	0.00	29	30	15	0	7.9459*	0.0188	Codominant
35	xmwc89	0.00	18	37	19	0	0.0270	0.8780	Codominant
36	xwmc161	0.00	20	36	17	1	0.2603	0.4820	Codominant
37	Lr19	0.00	20	40	14	0	1.4595	0.8901	Codominant
38	gpw4310	0.00	18	35	20	1	0.2329	0.7841	Codominant
39	xwmc232	0.00	17	36	21	0	0.4865	0.7841	Codominant
40	cfd23	0.00	20	34	20	0	0.4865	0.6402	Codominant
41	xbarc261	0.00	22	29	23	0	0.8919	0.7428	Codominant
42	xcfd43	0.00	19	34	21	0	0.5946	0.7428	Codominant
43	Gpw3254	0.00	18	40	16	0	0.5946	0.4407	Codominant
44	Lr13	0.00	14	41	17	2	1.6389	0.7841	Codominant
45	Lr26	0.00	20	38	16	0	0.4865	0.7841	Codominant
46	cfd71	0.00	19	34	21	0	0.5946	0.7428	Codominant

hmzA= Homozygous for P_1, hmzB= Homozygous for P_2, htz=Heterozygous F_1.

*, ** Significant at 5% a 1% levels respectively.

The calculated chi-square values were compared with tabulated values for 5% and 1% probability levels at two degrees of freedom. Out of 46 tests for 46 SSR, four test markers deviated significantly from expected ratios at both probability levels revealed that observed data were not agreement with expected ones for these four cases, while rest of the marker loci (42) revealed non-significant chi-square values when compared with table values indicating fulfillment of 1:2:1 segregation ratio (Table:1) Distorted segregation of molecular marker loci appears to be a common phenomenon in many crop species (Cloutier *et al.,* 1991; Yarnagishi *et al.,* 1996) [6, 31].

Construction of genetic linkage map for grain size and related traits

A new intra-specific genetic linkage map GW-11 (bold grain) X GW-322 (small grain) for cultivated bread wheat was constructed using software IciMapping v.4.1 (Meng *et al.,* 2015) [18]. A total of 46 polymorphic markers were integrated into a seven linkage groups (LGs) (Fig. 2). Linkage map of

seven linkage groups with a total map length of 77.31 cM was constructed using data from 46 marker loci for 74 F_2 progenies. The map lengths of individual linkage groups ranged from a minimum of 2.74 cM (LG2) to a maximum of 26.89 cM (LG3). The complete linkage map in the present study consisted of total 31 molecular markers distributed to seven linkage groups with a total length of map accounted 77.31 cM. The total number of markers was highest in linkage group 3 covering total map length of 26.89 cM. Linkage group 2 and linkage group 4 had the lowest number of markers (2 loci each) and linkage group 2 had lowest map length (2.74 cM) among seven linkage groups in the present study. Fifteen markers remained unlinked, probably due to insufficient genome coverage. The possible explanation for lower number of markers and short distance cover on genome could be the use of only single types of SSR markers which were in limited in number. Other alternative reasons could be the sizes of the mapping populations, genetic constitution of parental lines, and number and polymorphism of marker loci obtained for both parental lines. A similar result was also reported in a study of linkage map by Petsova *et al.*, (2002) [19] consisted of total 505 molecular markers which were distributed on 26 different linkage group with total map length of 1272 cM in wheat. The highest number of markers was in the linkage group 7 with total map length of 259 cM and linkage group 1 had lowest molecular marker number with shortest map distance 28 cM among all linkage groups.

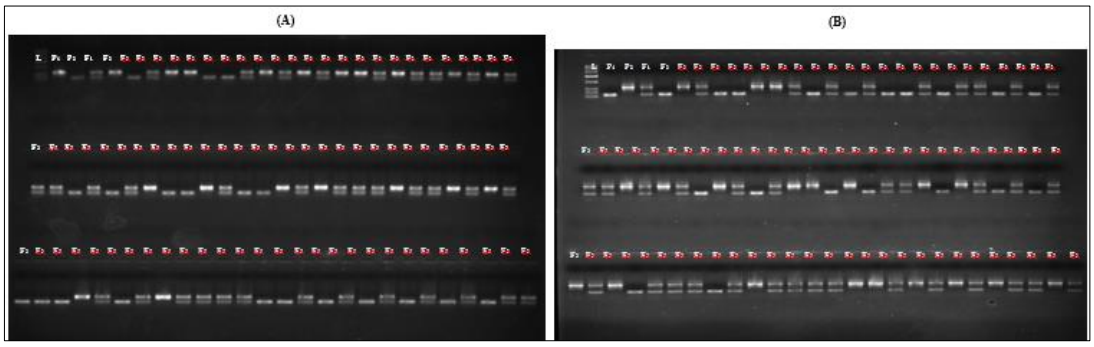

Fig 1: Agarose gel from genotyping of SSR loci (A) xgwm 508 (B) xgwm-149 markers differing in size of PCR-amplified in individual F_2 plants

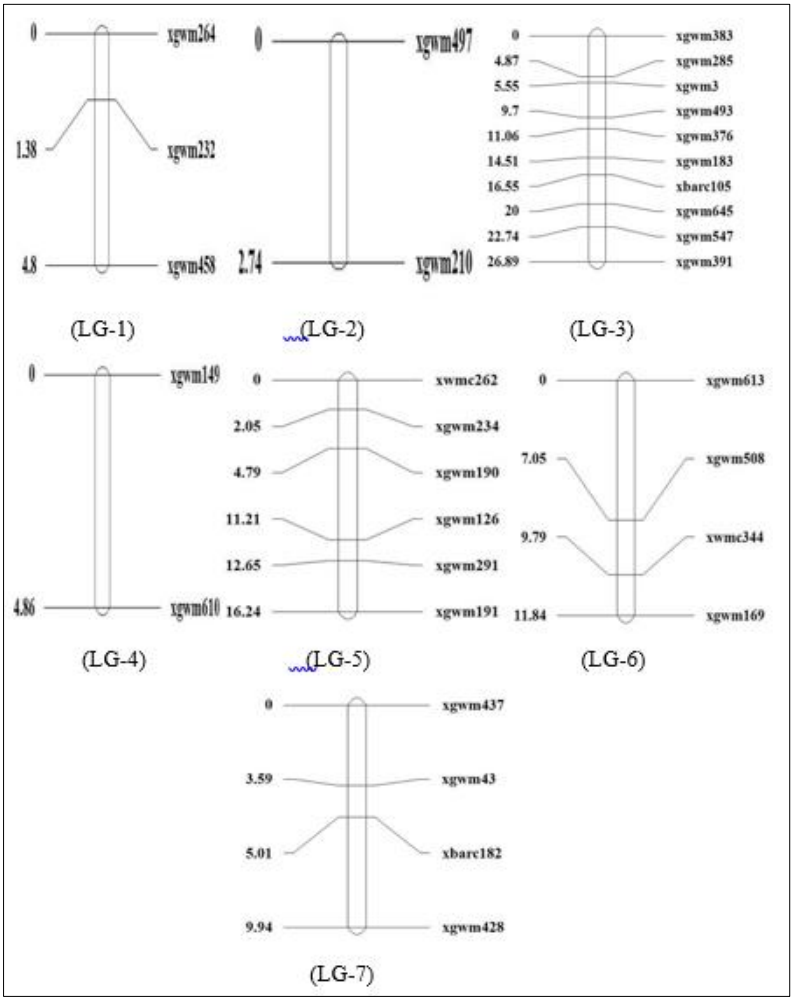

Fig 2: Genetic linkage group of bread wheat LG-1 to LG-7 indicated marker positions of chromosome No.1 to 7, respectively

QTL mapping for grain size and related traits

Genotypic data of 74 F_2 and phenotypic data obtained on 74 $F_{2:3}$ lines of the mapping population were analyzed for identification of the main effect QTLs using the software ICIM-ADD mapping in QTL IciMappingV4.1 (Meng *et al.*, 2015) [18]. The 77.31 cM linkage map constructed using Kosambi mapping function for 74 F_2 progenies from the cross GW-11 (bold grain) X GW-322 (small grain) QTL analysis was done for phenotypic data using 100-grain weight, number of grains per main spike, grain yield per plant, grain weight per main spike generated from a trial conducted at Wheat Research Station, Junagadh Agricultural University, Junagadh. QTL IciMapping was used for constructing linkage map was also used for QTL mapping. A linkage map output data file was used for the construction of QTL mapping. A total six QTL had been identified for grain size and related traits, one each for 100-grain weight (LG3 at 13 cM, LOD 6.68, 42.20 PVE%)(Tabel-2); number of grain per the main spike (NGPMS) (LG4 at 4 cM, LOD 3.28, 19.48 PVE%); grain yield per plant (GYPP) (LG5 at 14 cM, LOD 7.17, 36.25 PVE%); number of effective tillers per plant (NETPP) (LG7 at 3 cM, LOD 14.34, 59.17 PVE%); and two QTLs for grain weight per the main spike (GWPMS) (LG3 at 20 cM, LOD 4.64, 15.29 PVE% and LG6 at 11 cM, LOD 12.29, 58.03 PVE%)(Fig.3 and Fig.4).

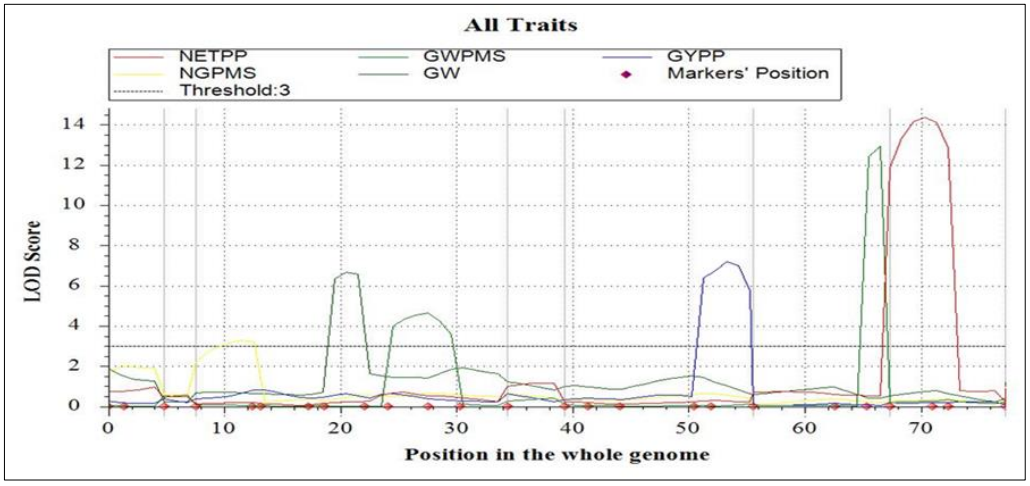

Fig 3: Position of grain size and related trait in whole genome with LOD score

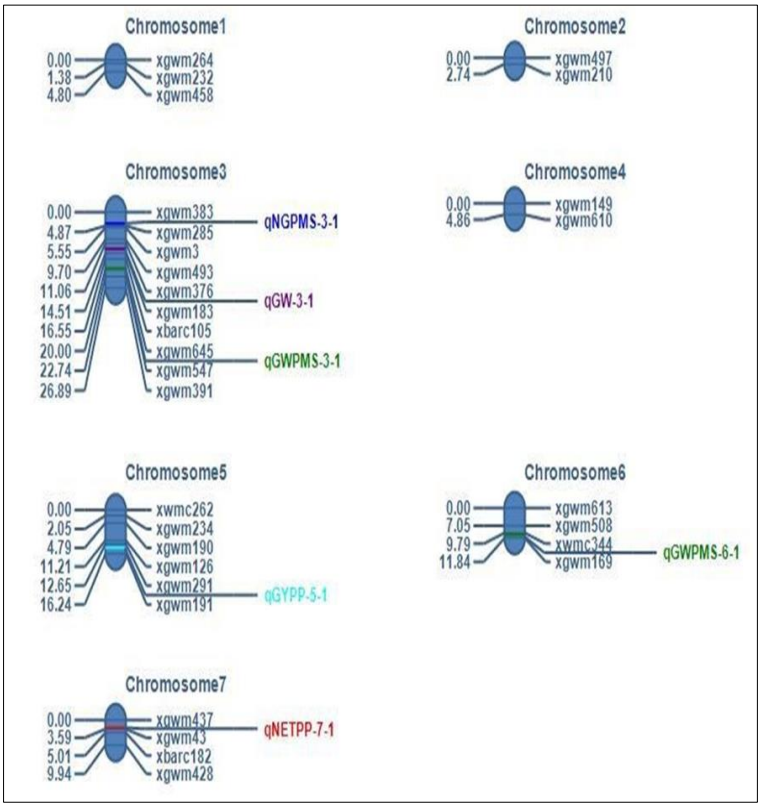

Fig 4: The position of grain size and related QTL in whole genome

Table 2: QTL identification for grain size and related traits with LOD score, PVE (%), additive and dominance effect

Trait ID	Trait Name	LG	Position	Left Marker	Right Marker	LOD	PVE (%)	Add	Dom
1	100-grain weight (GW)	3	13.00	xgwm376	xgwm183	6.68	42.20	-0.79	3.253
2	Number of grains per main spike (NGPMS)	3	4.000	xgwm383	xgwm285	3.28	19.48	-0.04	0.351
3	Grain yield per plant (GYPP)	5	14.00	xgwm291	xgwm191	7.17	36.25	-2.87	0.56
4	Grain weight per main spike (GWPMS)	3	20.00	xgwm645	xgwm547	4.64	15.29	-0.10	-0.02
5	Grain weight per main spike (GWPMS)	6	11.00	xwmc344	xgwm169	12.92	58.03	-0.19	-0.04
6	Number of effective tillers per plant (NETPP)	7	3.0	xgwm437	xgwm43	14.34	59.17	-3.36	0.35

With the help of molecular markers, more than 100 quantitative trait loci (QTLs) for GW in wheat have been identified to date using bi-parental populations or cultivar populations (Rustgi *et al*,. 2013; Maphosa *et al.*, 2014; Tyagi *et al.*, 2014) [22, 16, 28]. Many studies were done on QTL mapping for 100-grain weight which supported the present study; QTL positioning of 1000-grain weight was done by Wei *et al.* (2014) identified qTgw6A1, qTgw6A2, qTgw7A, qTgw2D2, qTgw6A1, qTgw1B, qTgw2A, qTgw2D1, qTgw6A with chromosome position 56.1, 62.2, 75.7, 51.8, 56.1, 42.6, 77.9, 25.4, 62.2.Likewise, Gao *et al.* (2015) [11] identified QTL named QKNPS-DH-3A-1.1 on chromosome 3 with apposition of 96 cM, PVE 6.88%, the additive effect of -2.10 for a number of grains per main spike which proved similar results with the present study. Assanga *et al.* (2017) [1] mapped quantitative trait loci for grain yield and its components and identified QTL named as Qmt.tamu.5A.1 on chromosome 5 at position of 100.4 cM which support findings of persent study for grain yield per plant. QTL mapping for grain weight per main spike was studied by many wheat researchers. Two QTL identified by Cui *et al.* (2012) [7] on chromosome 3 and 6 supported the findings of present study. Zhang *et al.* (2016) identified QTL named as QSN-DH-7A on chromosome 7 was online conformity with the present study for number of effective tillers per plant.

Conclusion

QTL mapping in plant breeding provides a valuable contribution to find out important traits in wheat. The detection of new QTLs associated with grain size should be useful for wheat genetic improvement in the future, especially as these QTLs appear to have relatively large effects. Ideally QTL associated with grain size found at chromosome number 3, 5, 6, 7 and the markers attached to the QTL after validation could have the potential to be used for molecular breeding. It can be routinely used by breeders in marker assisted selection in wheat breeding programs. Moreover, by adapting new and novel marker systems like EST-SSRs, SNPs etc., it could be possible to select best genotypes for breeding purpose in wheat.

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