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Genome-wide association mapping revealed SNP alleles associated with resistance to cereal cyst nematode (*Heterodera filipjevi*) in wheat

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Abstract

Resistance traits are economically important in crops in terms of accessibility to promising resistant germplasms. This study was conducted to evaluate SNP marker-trait association for cereal cyst nematode (CCN), *Heterodera filipjevi* in a large number of natural bread wheat populations. Phenotypic data analysed using GLM (Generalized Linear Model) indicated significant differences among the landrace accessions for resistance to *H. filipjevi*. The genotyping was performed by 152K SNP chip on 188 accessions. After filtering, 10,471 polymorphic SNPs were employed for Genome Wide Association Study (GWAS). Population structure among the wheat genotypes were investigated using 840 well distinct SNP markers. Two sub-populations were revealed by structure software, and eleven markers were found to be significantly (p-value < 0.001) associated with resistance to *H. filipjevi* on chromosomes 2A, 3B, 4A, 4B, 5A, 5B, 5D, and 6B. The linkage disequilibrium analysis for all significantly associated SNPs showed that markers on chromosomes 4A and 4B were in high intra-chromosomal linkage disequilibrium, and consequently, eight markers were recommended as strongly associated with resistance to *H. filipjevi*. The present study demonstrated valuable sources of resistance in the studied wheat genotypes against a widespread and important species of CCNs. The associated markers could be used in molecular breeding programs of bread wheat.

Keywords: Association mapping, *Heterodera filipjevi*, GWAS, SNP, Wheat.

INTRODUCTION

Cereal Cyst Nematodes, CCNs (*Heterodera* spp.) are one of the most important causal agents of yield losses on wheat annually, hence its global importance is known in most wheat-growing areas (Smiley *et al.*, 2017; Toumi *et al.*, 2018). The genus *Heterodera* is divided into nine groups based on morphological and molecular characteristics (Handoo and Subbotin, 2018), in which *H. filipjevi* is one of the most important species belonging to Avenae group. Host plants of *H. filipjevi* include wheat, rye, barley, corn, and many grasses (Smiley *et al.*, 2017). Yield losses caused by *H. filipjevi* in three winter wheat cultivars in Iran were estimated to be 20.4 to 24.8% (Karimipour Fard *et al.*, 2018). Wheat is one of the world's most commonly used cereal grains growing all over the world and feeding more than 40% of the world population. Amongst the different types of wheat grain, bread wheat (*Triticum aestivum* L., AABBDD) is the most economically important crop and the world's most widely cultivated cereal. It is originated from hybridization between *Triticum urartu* (AA) and *Aegilops speltoides*-related species (BB), forming *Triticum turgidum* ssp. dicoccoides, and again hybridized between *Triticum turgidum* ssp. durum (AABB) and *Aegilops tauschii* (DD), forming the modern hexaploid bread wheat (AABBDD).

Resistant cultivars are often regarded as one of the most effective tools for controlling CCNs. Many sources have been reported and reviewed for conferring resistance measures. Important sources of resistance genes were revealed in landrace varieties by identifying many resistance *Cre* genes. In recent years, different types of molecular markers have been applied in plants such as Restriction fragment length polymorphisms (RFLPs), microsatellites or simple sequence repeats (SSRs), expressed sequence tags (ESTs), cleaved amplified polymorphic sequence (CAPS), randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphisms (AFLPs), inter simple sequence repeat (ISSR), Diversity arrays technology (DArT) and single nucleotide polymorphism (SNP) (Dhingani *et al.*, 2015). In genetic studies, single nucleotide polymorphisms (SNPs) are one of the most effective tools. SNPs are more powerful in estimating population structure which are abundant in the genome.

In recent years, research on wheat genome recorded 90K SNP by the new Infinium to 500K and 4 M in Illumina shortgun WGS array (Avni *et al.*, 2014; Wang *et al.*, 2017; Lai *et al.*, 2015). Association mapping (AM), is known extremely for the identity of markers associated traits based on linkage disequilibrium (LD) in plants. AM has been applied to discovery of quantitative trait loci (QTL) on chromosomes in range of crop species. To date, QTL regions on different chromosomes were detected in association with particular traits using AM in wheat such as pre-harvest sprouting resistance, low α-amylase and seed color (Rabieyan *et al.*, 2022) and grain-associated traits (Wang *et al.*, 2017), resistance to CCNs (*Heterodera* spp.), resistance to root lesion nematode (*Pratylenchus* spp.) and resistance to crown rot (Dababat *et al.*, 2016; Erginbas-Orakci *et al.*, 2018; Kumar *et al*, 2021; Sohail *et al.*, 2022).

Several QTLs have been suggested to affect on resistance to *H. filipjevi*. The first survey of QTLs conferring resistance to *H. filipjevi* in wheat reported eleven QTLs on chromosomes 1AL, 2AS, 2BL, 2D, 3AL, 3BL, 4AS, 4AL, 5BL, 6B, 6D and 7BL (Pariyar *et al.*, 2016; Dababat *et al.*, 2021).

The aim of the present study was to *a*: find marker-trait associations within 188 wheat genotypes collected from West Asia-North Africa, WANA, *b*: identify SNPs associated with resistance to *H. filipjevi* in wheat, *c*: combine analyses of phenotypic data and association mapping.

MATERIAL AND METHODS

Plant Materials and Inoculum Preparation

A total of 223 wheat accessions originating mostly from West Asia and North Africa (WANA countries) with three wheat cultivars as susceptible and resistant controls were used to evaluate their resistance to *H. filipjevi*. It is worth to note that 188 accessions out of 223 accessions used for phenotyping indicated sufficient DNA quality for SNP calls. The wheat accessions were provided by the International Center for Agricultural Research in the Dry Areas (ICARDA), and were originated from Afghanistan (7), China (1), Iran (164), Iraq (3), Morocco (1), Pakistan (7) and Syria (5) countries. The pedigree of the 188 wheat genotypes used in this study is given in supplementary Table.1. For the preparation of inoculum, the collecting of nematodes, extracting, identifying, incubation of the cysts and obtaining infective juveniles were conducted as described by Majd Taheri *et al.*, (2019).

Phenotyping Assessment

The phenotypic evaluation was performed in a growth chamber at the Iranian Research Institute of Plant Protection (IRIPP). Wheat seeds of each accession, were sterilized, germinated and planted in a plastic tube filled with a mixture of sand, field soil, and organic matter (70:29:1, v:v:v) arranged in a completely randomized design with five replications. The wheat cultivars Bezostaya and Sonmez were chosen as the susceptible and resistant checks, respectively. Each plant was inoculated with 1 mL of inoculum containing 500 fresh second stage juveniles in a water suspension. After nine weeks, the level of resistance was counted and categorized into four groups based on the number of white females and cysts, Resistant (R) ≤ 3; Moderately resistant (MR) = 3–7; Susceptible (S) = 7–20; Highly Susceptible (HS) ≥ 20 according to Sharma *et al.*, (2013). Normality of data and Homogeneity of variances were examined with Shapiro-Wilk test and Levine’s test, respectively. All phenotypic data were analysed using Generalized Linear Model (GLM) using statistical software SAS v9.4 and mean separation was conducted using Duncan's Multiple Range Test.

Genotyping and Data Preprocessing

Genomic DNA was extracted from fresh leaves using a modified CTAB (cetyltrimethylammonium bromide) method as described by Saghai-Maroof *et al.* (1984). Samples were genotyped by genotyping-by-sequencing (GBS) and Diversity Arrays Technology (DArT) (Sansaloni *et al.*, 2011) using 152K SNP panel at the Genetic Analysis Service for Agriculture (SAGA) at the International Maize and Wheat Improvement Center (CIMMYT), Mexico. The quality of genotypic data were curated by removing SNPs with minor allele frequency (MAF) less than 0.05 and missing data more than 20% from the subsequent analysis (Bhatta *et al*. 2018), and the heterozygous data were considered as missing data (Mourad *et al*. 2018; Pariyar *et al*. 2016), which left a set of 10,471 polymorphic SNP markers with known chromosomal position (based on Chinese spring map of IWGSC RefSeq v1.0 assembly (Appels *et al*., 2018)) for further analysis.

Analysis of Population Structure

The 840 SNP markers were selected based on physical position on chromosomes (A, B and D) from the total 10,471 markers with known chromosomal positions. Population structure analysis was performed using a Bayesian model in software STRUCTURE v2.3.4 (Pritchard *et al.*, 2000), where number of populations (K) were assumed from 1 to 10 using 100,000 burn iterations followed by 100,000 Markov-Chain Monte Carlo (MCMC) iterations. Process was repeated 5 times for each K. Output was visualized using STRUCTURE harvester and the optimal K value was identified based on the LnP(D) and Evanno's ΔK (Evanno *et al.*, 2005).

Linkage Disequilibrium Association mapping

Linkage disequilibrium and Genome-Wide Association Study, GWAS were implemented using 10,471 SNPs with known chromosomal positions. Chinese Spring genome map IWGSC RefSeq v1.0 assembly was used as the reference genome (Appels *et al*., 2018). A mean pairwise r for the 21 chromosomes was determined. The LD heat maps plot for significantly associated SNPs was constructed by using Haploview software 4.2 (Broad Institute, Cambridge, MA). GWAS was conducted using the General linear model (GLM) and Mixed linear model (MLM) (Q+K) in TASSEL v. 5.2.51 (Bradbury *et al*., 2007). The Q matrix was adapted from the K=2 for association mapping for controlling spurious results due to population stratification as a major issue in GWAS. TASSEL software was employed to estimate kinship matrix and the association analyses were carried out to generate Manhattan and quantile-quantile plots (Q–Q plot). A threshold P-value of 0.001 (–log10P= 3) was applied to declare significant SNPs for marker-trait association results. The phenotypic variation (R^2) was estimated for significant markers. To reduce the false discovery rate, FDR was implemented at 0.001 level in SAS v 9.4 (SAS Institute Inc., Cary, NC, United States).

RESULTS AND DISCUSSION

Wild relatives of wheat are important sources of disease resistance. In recent years, different types of molecular markers have been applied to study the genetic traits in many crops i.e., barley (*Hordeum vulgare* L.), maize (*Zea mays* L.), potato (*Solanum tuberosum* L.), rice (*Oryza sativa* L.), soybean (*Glycine max* (L.) Merr.), sorghum (*Sorghum bicolor* L.) Moench), tomato (*Lycopersicon esculentum* Mill.) and wheat (*Triticum aestivum* L.). SNP chips were mostly applied in GWAS which makes it easier to identify QTLs associated with certain traits. Our raw data and variances were normal and homogeneous, respectively. The analyses of phenotypic data revealed significant differences among the accessions for resistance to *H. filipjevi* (Table 1). The 35 % of wheat accessions showed resistant (R) reaction to *H. filipjevi*, 44% of the accessions were moderately resistant (MR) and 21% were susceptible (S) (Figure 1). Most of the Iranian genotypes indicated moderately resistant (45%) trait (Majd Taheri *et al.*, 2019). Of the 10,471 SNPs found to be highly associated with resistance to *H. filipjevi*, 4,096 (39%), 4,739 (45%), and 1,636 (16%) SNPs were recorded on the AA, BB, and DD genomes, respectively (Figure 2). AA and BB genomes have a higher distribution of SNPs than the DD genome, this finding is in agreement with similar studies (Wen *et al.*, 2017; Gahlaut *et al.*, 2019; Rabieyan *et al.*, 2022; Tehseen *et al.*, 2022). The minimum number of SNPs were associated with resistance to *H. filipjevi* from chromosome 4D (147 SNPs) and most numbers of SNPs were from 2B (887 SNPs). Population structure analysis implemented using 840 markers, indicated two possible subpopulations, based on the clear peak at k=2 (Figure 3). The first and second group consisted of 62% and 38% of the wheat accessions, respectively. We found significant differences among the genotypes for resistance to *H. filipjevi*. The genetic diversity of wheat genotypes from our previous experiment revealed the suitability of this group of wheat genotypes for association mapping studies (Majd Taheri *et al.*, 2019).

Using 10,471 SNPs, linkage disequilibrium (LD) was determined by calculating squared correlation coefficient (r^2) for the 21 chromosomes. We applied a mixed linear model (MLM) and General linear model (GLM) in GWAS analysis. QQ-plots and Manhattan plots of the GWAS results of both GLM and MLM analysis were compared for resistance trait which are shown in Figure 4. Based on the obtained QQ-plot from GLM and MLM models, the Q-Q plot of GLM shows deviations from the slope line, demonstrating the loci which deviate from the null hypotheses and indicating significant positive marker-trait association which makes GLM as a better approach. Manhattan plots represent the profile of the P-value of SNPs in Figure 5. A total of 11 SNPs significantly associated with resistance to *H. filipjevi* trait and crossed the false detection rate (FDR) at $p < 0.001$ were identified. The phenotypic variation (r^2) explained by the individual SNPs ranged from 7 to 13% (Table 2).

So far, some significant Marker-trait associations (MTAs) were identified on wheat chromosomes to agronomic characteristics and diseases. This collection of wheat genotypes has not been utilized for resistance studies to cereal cyst nematode so far, however, GWAS of diverse panels against *H. filipjevi* was done by Pariyar *et al.*, (2016) and Dababat *et al.*, (2021). In the present study, 11 markers were significantly (p-value < 0.001) associating with resistance to *H. filipjevi* which were detected on chromosomes No 2A, 3B, 4A, 4B, 5A. 5B, 5D and 6B. The linkage disequilibrium (LD) analysis for all significantly associated SNPs showed that 3 markers on 4A and 2 markers on 4B Chromosomes were in high intra-chromosomal LD, hence the 11 SNPs could be reduced to 8. It is noteworthy that the D genome carries only one of all identified MTAs in this study, likely implies the low level of diversity in the D genome originated from the late hybridization of *Aegilops tauschii* during the evolution of common wheat (Gahlaut *et al.*, 2019). A previous GWAS have demonstrated 11 QTLs on chromosomes 1AL, 2AS, 2BL, 3AL, 3BL, 4AS, 4AL, 5BL and 7BL (Pariyar *et al.*, 2016). Another study identified QTLs on chromosomes 1A, 2A, 2B, 2D, 3A, 6B, and 6D were detected using a mixed linear model (MLM) (Dababat *et al.*, 2021). Fourteen genes for resistance to CCN have been identified which include the following: *Cre1*, *Cre2*, *Cre3*, *Cre4*, *Cre5*, *Cre6*, *Cre7*, *Cre8*, *Cre9*, *CreR*, *CreV*, *CreX*, *CreY* and *CreZ* (Ali *et al.*, 2019; Kishii, 2019; Dababat *et al.*, 2021). CCN resistance genes *Cre1*, *Cre2*, *Cre3*, *Cre5* (syn. *CreX*), *Cre6*, *Cre8* and *CreR* identified in wheat and its relatives on chromosome 2B, 2A, 2D, 2A, 5A, 6B and 6D, respectively (Slootmaker *et al.*, 1974; Asiedu *et al.*, 1990; Eastwood *et al.*, 1991; Delibes *et al.*, 1993; Jahier *et al.*, 1996; Paull *et al.*, 1998; Ogbonnaya *et al.*, 2001). Our results demonstrated three QTLs (on 2A, 5A and 6B) found on chromosomes with identified resistance genes. Surprisingly *Cre8* gene as a resistance gene to CCN, *H. avenae* was mapped by Williams *et al.*, (2003) on chromosome 6B, moreover the effective role of *Cre8* in conferring of resistance to CCN, *H. fili pjevi* in wheat was emphasized by Imren *et al.*, (2013). Our finding suggests that the marker identified in this study may be present in the genomic region of the *Cre8* gene, however further evidences are needed to confirm the exact loci. Similar to the present study some QTLs that confer resistance against other cereal nematodes were recently reported on wheat, *i.e*. in *H. avenae* on chromosomes 5A, 5B, 5D and 6B, in root lesion nematodes, *P. neglectus* on chromosomes 3B, 4A and 6B, *P. thornei* on chromosomes 2A, 3B and 5B (Dababat *et al.,* 2016).

Pleiotropic effect resistance genes to multiple races (HG types) of soybean cyst nematode (SCN), *H. glycines* and Root knot Nematode (*Meloidogyne incognita*) was revealed in soybean line 438489B which carrying multi-nematode resistance gene package (Vuong et al., 2011). Recently two QT controlling reniform nematode, RN (*Rotylenchulus reniformis*) resistance were identified in the SCN resistance gene *GmSNAP18* at the *rhg1* locus and its paralog *GmSNAP11* in soybean line 438489B (Usovsky et al. 2021). Hence, there is a possibility of similarities between identified QTLs which necessitates further experiments to determine common QTL between two or more pathogens. Importantly, it is obvious that a QTL with capability of inducing resistance to plant against several traits, is a valuable resource in breeding programs. In conclusion, in this study, 188 wheat accessions were applied to perform association mapping, and 10,471 SNPs used for GWAS after filtering according to the MAF, missing data, and heterozygous data. We estimated the phenotypic and genotypic parameters for resistance trait and eleven significantly associated SNP markers were detected by GLM. Based on the results, the use of populations from different genetic backgrounds provide further progress in identifying valid QTLs. The findings of present study demonstrated valuable sources of resistance in the studied wheat genotypes to a widespread and important species of CCN in some areas of the crescent fertile region for inclusion in future breeding programs by new resistance gene resources.

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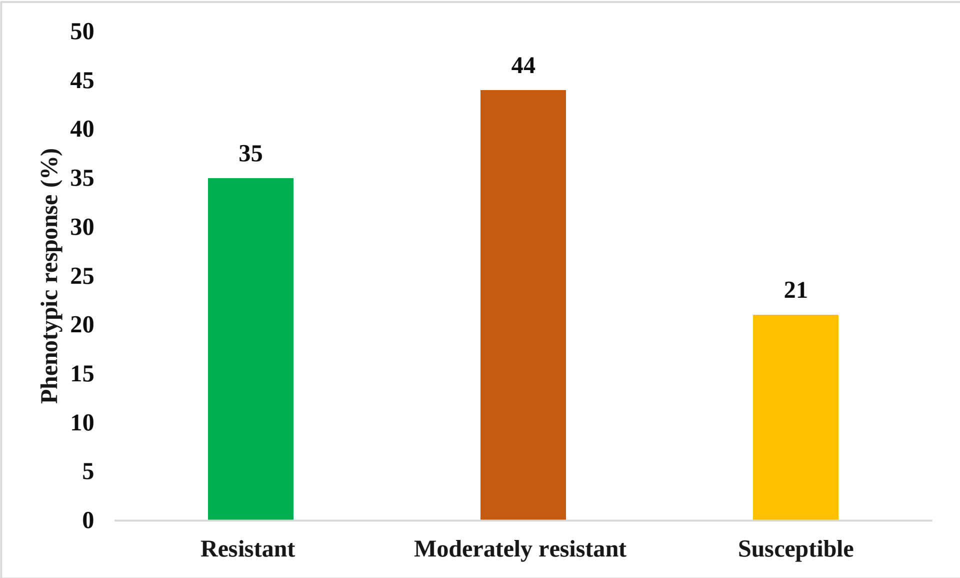

Fig 1. Phenotypic responses of wheat accessions to *Heterodera filipjevi* based on the number of white females and cysts (Resistant≤ 3; Moderately resistant= 3-7; Susceptible= 7-20).

Table 1. Analysis of variance of the reaction of wheat genotypes to *Heterodera filipjevi* using Generalized Linear Model (GLM).

Source	Degrees of freedom	Mean of square	F value	Pr > F
Genotype	225	1.15	3.60	<0.0001
Error	904	0.32	-	-
CV[a]	-	25.09	-	-

[a] Coefficient of variation.

Table 2. Single nucleotide polymorphisms (SNPs) significantly associated with resistance to *Heterodera filipjevi*.

No.	SNP Marker	CHR	POS (bp)	FDR	P value	Allele	Allelic effect	R^2 (%)	cM
1	3034005	2A	7919418	0.00060	0.00007	T/C	-0.49	10	8
2	1106119	3B	183514671	0.00070	0.00034	T/G	0.89	13	-
3	2262587	4A	374449717	0.00060	0.00017	C/T	0.42	10	-
4	1220611	4A	430870112	0.00070	0.00046	T/C	-0.41	8	-
5	2266236	4A	433757511	0.00070	0.00037	G/A	-0.40	8	27
6	1128101	4B	605941582	0.00100	0.00081	G/A	-0.49	8	-
7	1244896	4B	608261318	0.00060	0.00023	C/G	0.52	9	45
8	1098989	5A	480788416	0.00100	0.00097	G/C	0.57	7	51
9	1209179	5B	506087964	0.00060	0.00011	A/G	-0.91	9	48
10	2260283	5D	380840282	0.00100	0.00096	G/A	0.52	7	-
11	1091272	6B	79045627	0.00070	0.00051	A/G	-0.42	8	23

CHR: Chromosome; POS: Position; FDR: False Discovery Rate; R^2: Effect due to genetic variation: Cm: Centimorgan.

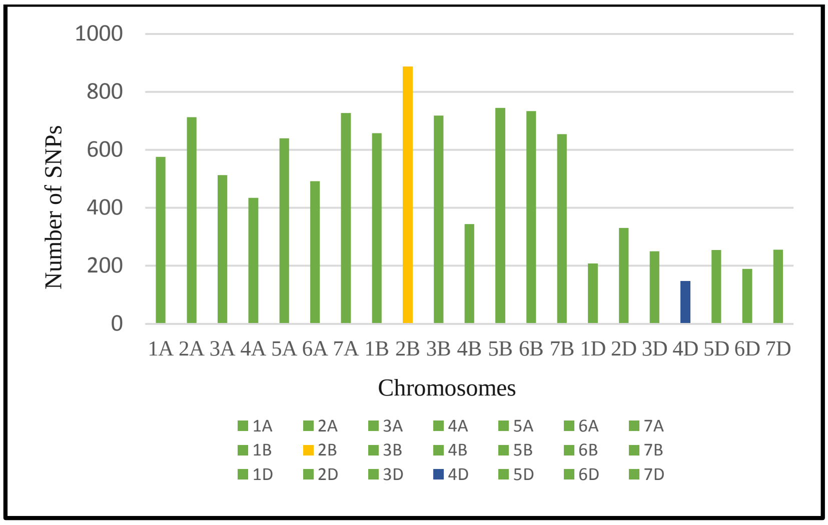

Fig 2. Genome origin (A, B and D) of tested wheat SNPs of tested wheat genotypes. Yellow and blue columns represent highest and lowest numbers of SNPs, respectively.

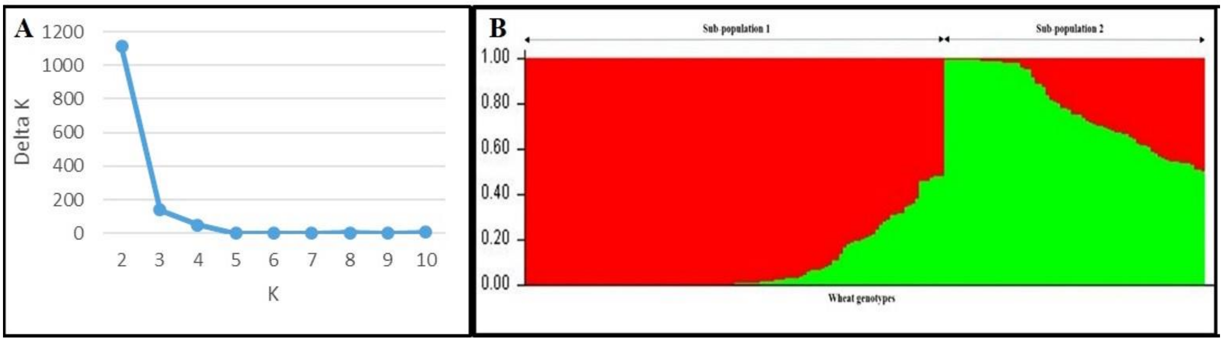

Fig 3. A: Graph of delta K values showing highest probability at number of groups (K= 2) and B: Estimated population structure of 188 wheat genotypes on k= 2.

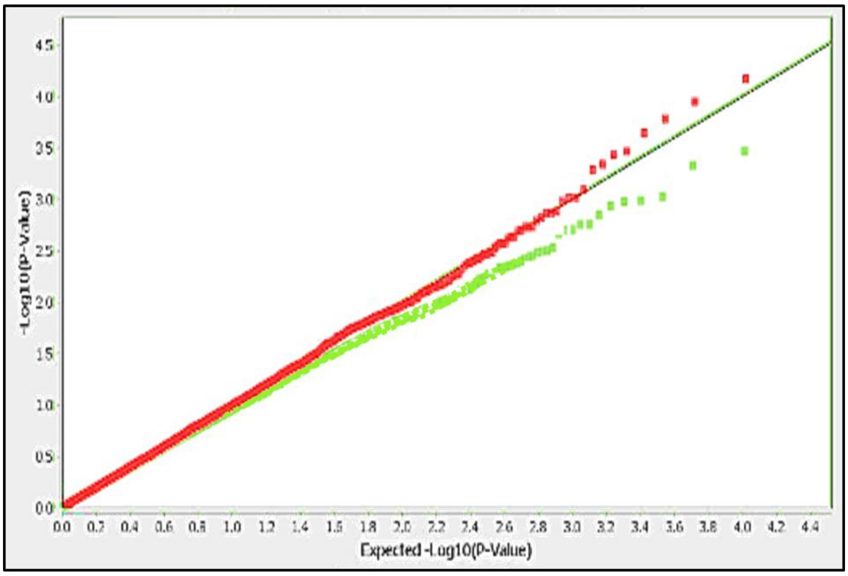

Fig 4. QQ (Quantile-Quantile) plots, Red line represents the observed P values using the GLM (Q) model and green line represents the observed P values using the MLM (Q + K) model.

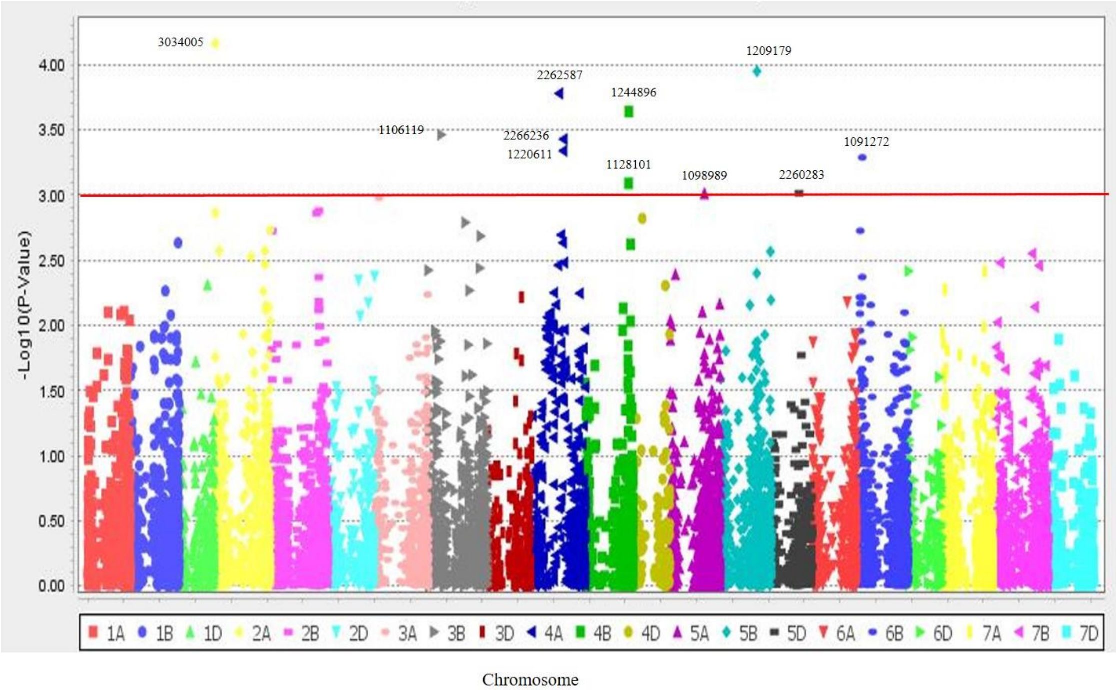

Fig 5. Manhattan plots of *P* values showing genomic region of wheat genotypes associated with *Heterodera filipjevi* resistance. The X-axis represents the position of markers over the wheat chromosomes and Y-axis represents -log10 (P-values) of the marker-trait association. Each Point in the plot represents a SNP marker. The red line represents the threshold for genome-wide significance. Markers with −log10 (P-values) above the threshold are candidates.

شناسایی آلل‌های SNP مرتبط با مقاومت گندم به نماتد سیستی غلات Heterodera filipjevi با استفاده از نقشه یابی ارتباطی گسترده ژنوم

چکیده

وجود صفت مقاومت در گیاهان از لحاظ دسترسی به ژرم پلاسم‌های مقاوم امید بخش حائز اهمیت اقتصادی است. این مطالعه به منظور بررسی ارتباط نشانگر- صفت در تعداد زیادی از جمعیت‌های گندم نان نسبت به نماتد سیستی غلات Heterodera filipjevi انجام شد. نتایج حاصل از تجزیه و تحلیل آماري داده‌های فنوتیپی با استفاده از مدل خطی تعمیم یافته (GLM) نشان داد، ژنوتیپ‌ها از لحاظ واکنش مقاومت به نماتد از اختلاف معنی‌داری برخوردار هستند. ارزیابی ژنوتیپی با استفاده از یک تراشه 152 K SNP صورت گرفت. پس از اعمال کنترل کیفیت روی مجموعه داده¬ها، تعداد 10471 نشانگر SNP برای نقشه‌یابی ارتباطی گسترده ژنوم (GWAM) استفاده شد. آنالیز ساختار جمعیت با استفاده از 840 نشانگرSNP، جمعیت مورد مطالعه را به دو زیر جمعیت طبقه بندی نمود. یازده نشانگر متعلق به هشت جایگاه ژنی به طور معنی داری (0/001 >p-value) در ارتباط با صفت مقاومت به نماتد روی کروموزوم¬های A2، 3B، 4A، 4B، 5A، 5B، 5D و6B شناسایی شدند. از میان 11 نشانگر شناسایی شده، سه نشانگر روی کروموزوم 4 Aو دو نشانگر روی کروموزوم 4 Bاز میزان عدم تعادل پیوستگی بالایی برخوردار بودند. لذا تعداد 11 نشانگر شناسایی شده، به هشت نشانگر کاهش یافت. مطالعه حاضر منابع ارزشمندی از مقاومت به نماتد سیستی غلات را در ژنوتیپ‌های گندم نشان داد. نشانگرهای مرتبط را می‌توان در برنامه‌های اصلاح مولکولی گندم نان استفاده کرد.