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Genetic analysis of novel resistance sources and genome-wide association mapping identified novel QTLs for resistance to *Zymoseptoria tritici*, the causal agent of septoria tritici blotch in wheat

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Abstract

Septoria tritici blotch (STB) caused by *Zymoseptoria tritici* is one of the most important foliar diseases of wheat causing significant yield losses worldwide. In this study, a panel of bread wheat genotypes comprised 185 globally diverse genotypes were tested against 10 *Z. tritici* isolates at the seedling stage. Genome-wide association study (GWAS) using high-throughput DArTseq markers was performed and further gene expression analysis of significant markers trait association (MTAs) associated with resistance to STB was analyzed. Disease severity level showed significant differences among wheat genotypes for resistance to different *Z. tritici* isolates. We found novel landrace genotypes that showed highly resistance spectra to all tested isolates. GWAS analysis resulted in 19 quantitative trait loci (QTLs) for resistance to STB that were located on 14 chromosomes. Overall, 14 QTLs were overlapped with previously known QTLs or resistance genes, as well as five potentially novel QTLs on chromosomes 1A, 4A, 5B, 5D and 6D. Identified novel resistance sources and also novel QTLs for resistance to different *Z. tritici* isolates can use for pyramiding and development of durable resistance cultivars in future wheat breeding programs.

Introduction

Common wheat (*Triticum aestivum* L.) is the most important cereal crop in the world and plays important role in the diets of humans and livestock. Average global wheat production was reported to be 766.4 million tons in 2019 (<http://www.fao.org/worldfoodsituation/csdb/en>). This makes wheat the third important crop in terms of production after maize and rice. Global wheat production can be negatively influenced by abiotic and biotic stresses. Septoria tritici blotch (STB) caused by *Zymoseptoria tritici* is one of the most destructive fungal diseases of wheat worldwide (Kema et al. 1996; Hardwick et al. 2001). The fungus causes expanded necrotic lesions from early-emerging leaves to flag leaves, and the overall damage caused by STB can result in grain yield losses up to 50% under optimal environmental conditions (Mehrabi et al. 2006; Goodwin, 2007; Kema and van Silfhout, 1997; Suffert et al. 2011). Under epidemic conditions, fungicide application is required to control STB, but this strategy is not adequately effective due to quick adaptation of the pathogen to fungicides by sexual recombination and mutation (Torriani et al. 2009; Mohammadi et al. 2017; Kema et al. 2018). In addition, the application of fungicides has severe threats to human health and the environment. Therefore, the characterization of new resistance sources and the development of resistant wheat cultivars is the most economical and environmentally-friendly approach and fundamental strategy in breeding programs for sustainable agriculture and food security (Talebi et al. 2010; Mehrabi et al. 2015). Up to now, 22 major genes and 89 QTLs for resistance to *Z. tritici* have been reported and mapped on the wheat genome along with their closely linked markers (reviewed by Brown et al. 2015; Yang et al. 2018).

Both qualitative and quantitative resistance has been reported for resistance to STB in wheat. Qualitative resistance is usually controlled by major genes that confer complete resistance and follows by the gene-for-gene model (Brown et al. 2015). This model has been reported for the first time on *Z. tritici* isolate IPO323 and cv. Flame (Kema et al. 2000; Brading et al. 2002). Quantitative resistance is controlled by many minor genes and widely reported in wheat cultivars at both seedling and adult growth stages (Arraiano and Brown, 2006; Chartrain et al. 2004; Goodwin, 2007).

The qualitative resistance has been shown to be effective strategy for controlling different fungal diseases in wheat, but on the other hand, the rapid evolution and adaptation of *Z. tritici* populations result in overcoming resistance in most improved cultivars (Cowger et al. 2000; Stukenbrock et al. 2007; Makhdoomi et al. 2015; Muqaddasi et al. 2019). Therefore, in order to increase the durability of wheat resistance to STB, combining qualitative and quantitative resistance

genes is required to preserve resistance effectiveness over time (Brown et al. 2015; Vagndorf et al. 2017). Availability of high-definition genotyping using genotype by-sequence (GBS) technologies enables breeders to identify resistance genes in diverse germplasm that can be employed for the development of new resistant cultivars using the gene pyramiding approach (Kidane et al. 2017; Vagndorf et al. 2017; Arraiano and Brown, 2017; Muqaddasi et al. 2019).

The genetic architecture of resistance to STB has been mostly evaluated in different bi-parental populations for detection of large-effect genes or quantitative trait loci (QTLs) (Chartrain et al. 2004, 2005a,b,c; Simon et al. 2005; Tabib Ghaffary et al. 2011, 2012). Genome-wide association mapping (GWAS) using a large number of markers with high genome coverage is a powerful tool for detecting the resistance loci associated with diverse germplasm possessing natural variation of resistance genes (Bartoli and Roux, 2017). Various GWAS studies have been performed on diverse wheat germplasm (Mirdita et al. 2015; Vagndorf et al. 2017; Kidane et al. 2017; Muqaddasi et al. 2019; Arraiano and Brown, 2017). Most previous GWAS studies for revealing QTLs against STB have been done for detecting resistance loci against a mixture of isolates under natural field conditions. Therefore, GWAS analysis for specific isolate resistance using globally diverse pathotypes of *Z. tritici* is of great interest for breeders to identify new genes/loci that can be used in wheat breeding programs.

The present study relies on genotyping and phenotyping of a globally diverse panel of 185 wheat genotypes that were evaluated at the seedling stage against ten *Z. tritici* isolates (collected from Iran, Algeria, Turkey, France and The Netherlands). DNA fingerprinting of the wheat genotypes was performed using high-throughput DArTseq technology (SilicoDArT and SNP markers) aiming to identify and localize possible novel QTLs associated with isolate-specific resistance against *Z. tritici* in wheat.

Materials And Methods

Plant material and evaluation of STB infection

A total of 185 wheat genotypes comprised commercial cultivars, breeding lines and landraces with a wide range of genetic background was used in this study (Supplementary Table S1). The germplasm set included (1) 64 Iranian cultivars and breeding line that mainly used in wheat growing zones in Iran and also in national wheat breeding programs, (2) 103 landraces genotypes from globally diverse origins (20 countries) and (3) 18 wheat differential lines with previously known STB genes. To ensure the purity of the seeds, all landraces were grown in Iranian Seed and Plant Improvement Institute (SPII) field station and each genotype was selected from a single-spike.

Phenotypic evaluation of wheat genotypes against ten *Z. tritici* isolates (Table 1) was described in detail by Mahbubi et al. (2020). Briefly, STB isolates were pre-cultured in yeast-extract glucose (YG) liquid medium, and then these pre-cultures were used to inoculate 100 ml YG media per isolate. YG media were placed in an orbital shaker (set at 125 rpm) and incubated at 18°C for 5-6 days. The propagated yeast-like spores were collected and their concentrations were adjusted to 10^7 spores/ml (Abrinbana et al. 2012). Five seeds of each genotype were sown in plastic pots and 10-day old seedlings were spray-inoculated using hand sprayers. Inoculated plants were kept in dark plastic bags for 48 h at 20-25 °C and then transferred to a greenhouse with the environmental condition of 18-22 °C and >85% humidity (Kema et al. 1996; Makhdoomi et al. 2015). After 21 days post-inoculation, percentage necrotic leaf area covered with pycnidia of the first leaf for each genotype (at least three plants per pot) was scored visually as described before (Kema et al. 1996; Ghaneie et al. 2012).

Experiments were set up with a randomized complete block design with three replications.

Phenotypic data analysis

Data were normalized using arcsin square root-transformation (Sokal and Rohlf, 1995). Normalized data were analyzed using a linear mixed model (LMM), in which the isolates and wheat genotypes were the main effects and their two-way interaction as fixed effects. The BLUE value for disease severity (% of leaf area covered by pycnidia) for all isolates was calculated using the PROC MIXED procedure in SAS v9.3 (SAS Institute Inc., Cary, NC, USA) considering the genotype as a fixed effect and replication (block) considered as random effect. These BLUE values were then used for broad sense heritability estimates and correlations between isolates, cluster analysis of wheat genotypes, and also to perform GWAS (Godoy

et al. 2018). Cluster analysis of wheat genotypes according to pycnidia coverage data by each isolate was performed using the un-weighted pair-group method (UPGMA) and the dissimilarity matrix was measured using the Ward's method implemented in PAST software (Hammer et al. 2001). Pearson correlation coefficients among 10 STB isolates were calculated for the percentage of leaf covered by pycnidia in 185 wheat genotypes.

Genotypic data

DNA was extracted from seedling plants of each genotype using the CTAB protocol (Lasner et al. 1989). The quality and quantity of DNA were checked by spectrophotometer, and DNA concentration was adjusted to 100 ng/μl. DNA samples were plated in 96-plex and shipped to DArT Pty Ltd, Canberra, Australia, and genotyped using the DArTseq technology (Sansaloni et al. 2011; Alam et al. 2018). The detailed methodology for the generation of DArTseq markers (SNP and SilicoDArT) was described in Egea et al. (2017). We received a total of 94535 (54309 SilicoDArT and 40225 SNP) markers, which were polymorphic across 185 wheat genotypes. The physical position of markers obtained by aligning to the reference genome of to the reference genome of Chinese Spring (CS) IWGSC Ref Seq v1.0 (IWGSC et al. 2018).

Marker loci with unknown chromosome positions (based on genome assembly) were removed from the analysis, and the remaining markers were filtered using a minimum minor allele frequency (MAF) of 0.05 in TASSEL v.5.2.37 software (Bradbury et al. 2007). Wheat is a self-pollinated crop and we assumed that all genotypes are homozygous. Therefore, markers showing heterozygous were indicated as missing and markers with >20% missing were excluded. In total, 21773 (15856 SilicoDArT and 5917 SNP) distributed across the 21 chromosomes were maintained for analysis (Supplementary Table S2).

Linkage disequilibrium and population structure analysis

Linkage disequilibrium (LD) for DArTseq markers was implemented in TASSEL v.5.2.37. The critical r^2 -value was determined by root transforming the unlinked r^2 -values and taking the 95th percentile of the distribution as the threshold beyond which LD is likely caused by genetic linkage (Nielsen et al. 2014; Monostori et al. 2017). The graphical LD decay was imputed by the GAPIT R package (VanRaden, 2008; Lipka et al. 2012). Population structure was performed in STRUCTURE 2.1 based on an admixture model (Evanno et al. 2005). Model run as the burn of 10000 iterations followed by 10000 Markov Chain Monte Carlo iterations was set for accurate parameter estimates. The optimal value of K ranging from 1 to 10, with three independent runs. Principal coordinate analysis (PCoA) and cluster analysis among the wheat genotypes estimated in DARwin ver. 5.0 software using the Unweighted Neighbor-Joining (UNJ) algorithm.

Genome-wide association analysis

Genome-wide association mapping (GWAS) was conducted using 21773 DArTseq markers and the best linear unbiased estimates (BLUE) for phenotypic scoring data of 10 *Z. tritici* isolates. The R package Genome Association and Prediction Integrated Tool (GAPIT) (Lipka et al. 2012) was used for GWAS by fitting four different models such as mixed linear model (MLM), multiple loci mixed model (MLMM), compressed mixed linear model (CMLM) and Fixed and Random Model Circulating Probability Unification (FarmCPU) methods to select the best fitting model and reduced false discovery rate (FDR). Between the tested model, the FarmCPU was highly efficient in computation for complex traits by eliminates confounding issues arising due to population structure, controlling false positive and reduce their effect in GWAS analysis (Gahlaut et al. 2019; Kaler et al. 2020). In order to detect the marker-trait association (MTA) in GWAS, if the significant markers cross the false discovery rate threshold ($P = 0.05$), the significance threshold level of p-value $> (-\log_{10} P=4.00)$ was selected for MTAs considering the deviation of the observed test statistics values from the expected test statistics values in the Q-Q plots.

Map positions of significant MTAs were determined according to their genetic positions in a high resolution DArT-seq consensus map version 4, includes 105122 markers distributed across the 21 hexaploid wheat chromosomes (<https://www.diversityarrays.com/technology-and-resources/geneticmaps/>) and bread wheat IWGSC RefSeq v1.04 with BLAST+ v2.7.1 (Camacho et al. 2009). Overlapping markers on the same chromosome for resistance to different STB isolates were considered to tag a single QTL if their positions were closer than 10 cM and showed LD $r^2 > 0.3$. Then for comparison of the QTLs identified in this study, the map position of significant markers in each QTL was projected onto the two different wheat SSR consensus maps (Somer et al. 2004; Maccaferri et al. 2015) for cross-reference with previous SSR maps. Each QTL considered new if its position was ≥ 10 cM from previously reported STB resistance genes or QTLs.

Candidate genes identification and expression analysis

Promising candidate genes for individual MTAs were identified by aligning the physical position of markers to the sequence of the wheat genome assembly IWGSC v.1.0 (https://plants.ensembl.org/Triticum_aestivum/Info/Index). High-confidence annotated genes were retrieved from a ± 2 -3 Mb window of left and right of each identified MTA. For the expression analysis of annotated candidate genes (CG), the transcripts per kilobase millions (TPM) values for every CG were retrieved from the public wheat expression database at <http://wheatexpression.com> (Ramírez-González et al. 2018) and the Log transformed (Log2X) value was used to generate a heatmap using online tool ClustVis (Metsalu and Vilo, 2015).

Phenotypic data analysis

Analysis of disease severity data showed significant differences ($P \leq 0.001$) among wheat genotypes for resistance to *Z. tritici* (Table 2). Genotype \times isolate interaction was highly significant ($P \leq 0.001$) and indicated the differences in wheat genotypes for their responses to *Z. tritici* isolates. Heritability values for disease severity were high for all isolates. The high heritability values indicated that there was a limited replication variation relative to genotypic variation for all isolates. This is supported by high Pearson correlation coefficients for disease severity between all isolates (Table 3). The Pearson correlation coefficient between STB isolates ranged from 0.26 (IPO02166 and IPO92034) to 0.90 (IPO323 and IPO86013) with an average value of 0.61 (Table 3). In total, 239 isolate-specific resistances were found among all interactions ($n=1850$), of which 183 showed disease severity $P \leq 5\%$ (highly resistance), 56 were disease severity $5 < P \leq 10\%$ (resistance) (Supplementary Table S1).

The mean disease severity (DS) among wheat genotypes ranged from 0% (M3 synthetic) to 68% (KC4821 from Iran). Among 64 Iranian commercial cultivar and lines, 51 cultivars (80%) were susceptible to all isolates, while ER-M-92-20 was resistant to all isolates. In addition, Saison, Gaspard and Naaz showed resistance responses to all isolates, except for IPO02166. In total, 39 isolate-specific resistances were found among all interactions ($n=640$). The overall mean disease severity among Iranian commercial cultivar and lines was 45% ranging from 2% (ER-M-92-20) to 62% (Parsi). Among 103 landrace genotypes, 80 genotypes were susceptible to all isolates. The landrace IPK40740 (France) showed immune responses to all isolates and in addition five landraces (IPK45227, IPK46116, IPK40793, IPK41079 and IPK16452) were resistance to nine isolates. Overall, the mean disease severity of wheat landraces ranged from 0.9 to 68% with an average value of 44% and in total, 116 isolate-specific resistances were found among landrace-isolate interactions ($n=1030$). Cluster analysis and principal component analysis using omitted data from the percentage of leaf area covered by pycnidia, grouped wheat genotypes in three distinct clusters (Fig. 1). The first cluster comprised 63 wheat genotypes, of which 19 genotypes were Iranian cultivar and breeding lines, 7 genotypes with previously known *Stb* genes (Bulgaria, Israel 493, Cs-Synthetic, Shaflr, Es-Federal, M6 and Balance) and the remaining genotypes were from different sources. Resistance spectra of genotypes for this cluster ranged from 30.98% (IPO323) to 56.61% (IPO02166) (Table 4). Cluster-II contained 85 genotypes, with 32 Iranian cultivars, one differential cultivar (Curtot) and 52 genotypes from diverse origins. All genotypes in this cluster were highly susceptible to most of the isolates and the mean disease severity ranged from 45.14% (IPO323) to 66.57% (IPO02166) (Table 4). Cluster III comprised 37 genotypes, most of the differentials with known *Stb* genes (Veranopolis, Tadinia, Kavkaz-K4500, TE9111, Salamon, Arina, Riband and M3) grouped in this cluster. In general, genotypes from this cluster showed low mean disease severity ranged from 7.76% (IPO323) to 43.59% (IPO02166) (Table 4). Cluster-III comprised of 37 genotypes, including 13 Iranian cultivar and breeding lines, eight differentials (Veranopolis, Tadinia, Kavkaz-K4500, TE9111, Salamon, Arina, Riband and M3) and 16 landraces from different origins. In general, genotypes grouped in this cluster had a low mean disease severity ranging from 7.76% (IPO323) to 43.59%

Population structure and linkage disequilibrium

Extensive genotyping on 185 wheat genotypes resulted in 21773 (15856 SilicoDART and 5917 SNP) markers. The Unweighted Neighbor-Joining cluster analysis (Fig. 2a) and Bayesian model-based structure analysis (Fig. 2b) grouped wheat genotypes into four distinct subpopulations. Sub-population 1 (50 genotypes) consisted of 17 Iranian landraces and genotypes from Western Asia and Eastern Europe (Turkey, Tajikistan, Romania, Hungary). Sub-population 2 (29 genotypes) contained genotypes from diverse origins and two Iranian cultivars. Sub-population 3 (86 genotypes) comprised most of the Iranian improved genotypes (45 genotypes) and eight wheat differentials for resistance to STB. Sub-population 4 (20 genotypes) consisted of landraces accessions from different origins. In general, there was no significant relationship between cluster grouping and origin of wheat genotypes, most probably due to the ancient and recent international exchange of germplasm, while the Iranian improved cultivars mainly grouped in the same cluster. Several of the genotypes used in this study have been utilized as parental lines or have the same background pedigree. Therefore, a mixture of origins was observed in all clusters. Nevertheless, a clear distinction on the above-mentioned four main subpopulations was clearly observed based on molecular data analysis. Principal component analysis (PCA) was used to confirm the results of population structure and this also showed a distinct pattern of subpopulations. The first two PCs represented 66.72% of the total variation (Fig. 2c). A comparable result similar to cluster analysis and PCA was also observed by the heatmap plot of kinship matrix where four distinct clusters were identified (Fig. 3). Different subpopulations showed different resistance levels for most of the isolates. Subpopulation 1 (mostly of Iranian landraces and landraces from West Asia) has the highest susceptibility (mean DS=48.2), followed by the subpopulation 2 (mean DS=43.4) and subpopulation 3 and 4 (mean DS are 43.3 and 40.4, respectively) (Fig. 4). These associations between population structure and STB resistance indicated that corrections for population structure were required for association mapping analysis. In general, the PCA analysis was consistent with the results of STRUCTURE analysis, while the cluster analysis showed more consistence with subpopulations identified by STRUCTURE analysis (Fig. 2).

In LD analysis, the square of the correlation coefficient of alleles between loci (r^2) was not significant for most of the pairwise comparisons, whereas out of 1048575 marker pairs only 297546 (28%) intra-chromosomal pairs showed a significant level ($P<0.001$) of LD. Marker pairs on the genome-B showed a higher number of significant pairs in comparison to the genome-A and genome-D. Mean and critical r^2 values were 0.09 and 0.16, respectively. LD declined with increasing physical genetic distances between markers and r^2 value falling below the critical value over distances of 1.6 kb (Fig. 5).

Genome-Wide Association Analysis

Among different GWAS models, FarmCPU model shows reliable results and presented low spurious associations. For the analysis, phenotypic data (percentage of necrotic leaf area covered by pycnidia) and 21773 mapped DARTseq markers on 185 wheat genotypes were used. The highest number of markers mapped on A genome (8031) and B genome (9537) compared to those mapped on D genome (4205). Association analysis was performed separately for each isolate. A total of 27 significant MTAs were identified for STB resistance on 14 chromosomes (Table 5). Manhattan plots for the association between markers and STB responses to ten *Z. tritici* isolates were displayed in Figure 6 (Supplementary Figure 1).

The QTLs identified for different isolates but located at overlapping genomic region on a chromosome were considered a single QTL and assigned the same name using the nomenclature *Qstb.iau-* followed by the number of QTL in chromosome order (Table 5; Supplementary Figure S2), and finally 19 significant genomic regions associated with resistance to *Z. tritici* were detected on 14 chromosomes (Table 5). Most of the significant MTAs showed small effects and regarding significant associations ($P<0.0001$), only the MTAs that explaining > 5% of the phenotypic variations are presented in Table 5.

The position of these QTLs was compared to the position of mapped QTLs and known genes reported in previous studies (Summarized in Supplementary Figure 2). Overall, 13 QTLs were associated with isolate specific resistance, although 6 QTLs were associated with resistance to multiple isolates were on chromosome 2B (*Qstb.iau-3*), 2D (*Qstb.iau-5*), 3B (*Qstb.iau-6*), 4A (*Qstb.iau-7* and *Qstb.iau-8*) and 6B (*Qstb.iau-15*) (Table 5).

The corresponding locations of the identified QTLs were checked on a consensus map and their overlaps compared with previously known STB resistance genes and QTLs. Out of 19 QTLs, 15 QTLs identified in overlap regions or very close (≤ 10 cM) to genomic regions of the previously reported QTLs/genes. Five genomic regions were identified as putatively new QTLs for resistance to STB on chromosomes 1A, 4A, 5B, 5D and 6D (Supplementary Figure 2). Among these putatively novel genomic regions, *Qstb.iau1*, *Qstb.iau-11*, *Qstb.iau-13* and *Qstb.iau-16* were specific-isolate resistance QTLs for isolate RM6 (Iran), IPO02166 (Iran), IPO86013 (Turkey) and IPO98022 (France), respectively, and interestingly the *Qstb.iau7* were associated with resistance to four isolates (IPO98022, IPO99031, RM6, RM183) (Table 5).

Putative candidate gene identification and expression analysis

All the MTAs associated with resistance to STB isolates identified by FarmCPU method were mapped to the wheat physical genome. The physical reference genome of Chinese Spring cv. was used to survey the genes in the flanking regions on each MTA (IWGSC RefSeq v1.04 with BLAST+ v2.7.1). For each MTA, 5 Mb regions toward the left and right side was used to identify the putative candidate genes. A search for candidate genes resulted in identification of multiple genes, overall, 24 genes were found to have putative role in diseases resistance on the basis of literature (Table 6). These genes involved in different biological activities like protein kinase-like domain, Cytochrome P450, leucine-rich repeat domain superfamily, Fbox domain and Homeobox-like domain superfamily (Table 6).

Gene expression analysis for 24 candidate genes were conducted using RNA-seq expression data retrieved from Wheat Expression Browser (<http://www.wheat-expression.com/>). The results indicated for expression of 10 of the 24 genes that expressed at different developmental stages under diseases stress like as septoria, fusarium and leaf rust (Supplementary Figure 3). Five genes (TraesCS2A02G582500, TraesCS3B02G348200, TraesCS5A02G367700, TraesCS5B02G380400 and TraesCS6D02G327400) had high expression (up to 3 Transcripts Per Million; TPM) at different

developmental stages of wheat under septoria tritici blotch, leaf rust and fusarium head blight diseases and interestingly all these genes belonged to protein kinase-like domain superfamily (Supplementary Figure 3). Some candidate genes like as TraesCS1A02G004400, TraesCS2B02G001600 and TraesCS2D02G029700 expressed uniquely only in reproductive stage (Supplementary Figure 3).

Discussion

Novel sources for resistance to *Z. tritici*

Z. tritici is one of the most important foliar diseases in many wheat-growing areas, including Europe, Northern America and Asia (Hardwick et al. 2001; Mehrabi et al. 2015). The use of genetic resistance is the most appropriate strategy to control the disease. However, the rapid adaptation of *Z. tritici* populations leads to a quick breakdown of resistance. Thus, continuous characterization and utilization of new sources of resistance in breeding programs are prerequisites (Abrinbana et al. 2010; Ghaneie et al. 2012). Iran is one of the primary centers of origin of wheat, and it is proposed that the co-evolution of wheat and *Z. tritici* occurred in this region. Therefore, the characterization of wheat genotypes using isolates from this region is likely required to add new resources to gene pool, which can be used for pyramiding resistance genes (Ghaneie et al. 2012; Makhdomi et al. 2015; Aghamiri et al. 2015). We have recently studied the interactions of 185 wheat genotypes against ten *Z. tritici* isolates from different sources (Mahboubi et al. 2020). Most wheat genotypes showed were susceptible to all isolates. High broad-sense heritability suggested that the resistance variation is heritable, which are in agreement with previous studies on septoria resistance in different wheat germplasm both at seedling and adult plant stages (Dreisigacker et al. 2015; Muqaddasi et al. 2019; Riaz et al. 2020).

Among the isolates, IPO323 showed the highest number of incompatible interactions ($n=35$), while IPO02166 (originated from Iran) showed a high level of aggressiveness on wheat genotypes. All the differential wheat genotypes (except M3, Riband, Arina and Kavkaz-K4500) possessing known *Stb* genes were susceptible to most of *Z. tritici* isolates, which are in agreement with previous reports for the ineffectiveness of known *Stb* genes against *Z. tritici* populations (Abrinbana et al. 2012; Makhdomi et al. 2015; Mahboubi et al. 2020). Interestingly, two genotypes (ER-M-92-20 and IPK40740) and four differentials (Kavkaz-K4500, Arina, Riband and M3) were resistant to all isolates. These genotypes could be of interest as resistance sources that contain resistance genes or a combination of diverse yet-unknown *Stb* genes. Previous reports indicated that *Stb6* was ineffective against studied isolates (Mahboubi et al. 2020). Therefore, it can be concluded that the high resistance pattern of these genotypes should be due to the presence of *Stb15* or new unknown resistance genes. Besides these highly resistant genotypes, five genotypes (IPK45227, IPK26116, IPK41079, IPK16452 and IPK40793) showed resistance to nine isolates and can be used as valuable resistance sources in wheat breeding programs. Among wheat genotypes, M3 showed highly resistance (immune) responses to all isolates. This genotype contains *Stb16* and *Stb17* (Tabib Ghaffary et al. 2012; Mahboubi et al. 2020). Therefore, it can be concluded that this gene still is effective against STB, which is consistent with previous reports (Hosseinneshad et al. 2014; Makhdomi et al. 2015). The resistant genotypes identified in this study are likely novel sources of resistance, which can be used in breeding programs for the development of modern wheat cultivars.

QTL validation and alignment to previously reported STB genes and QTLs

In line with our previous study, we used GWAS analysis to identify novel QTLs against *Z. tritici* isolates. This approach enables breeders to enhance crop genetic improvement by incorporating suitable QTLs into wheat breeding programs (Ibrahim et al. 2020). To this aim, DArTseq markers were successfully used to genotype a globally diverse wheat germplasm. The use of high-density markers with broad genome coverage in GWAS improved the accuracy of identified QTLs for resistance to STB, which is a highly quantitative disease trait with a minor contribution of each QTL (Mirdita et al. 2015; Muqaddasi et al. 2019). Overall, we found 19 QTLs for resistance to STB that were located on 14 chromosomes (Table 5). The phenotypic variation explained by each QTL mapped on different chromosomes was relatively low ($R^2 \leq 0.17$), suggesting that the resistance to this pathogen follows a highly quantitative nature, which is consistent with previous reports (Kidane et al. 2017; Arraiano and Brown, 2017; Yates et al. 2019).

The precise comparisons of these QTLs with known QTLs were difficult due to different populations, isolates and markers used elsewhere. However, using consensus wheat maps, it was possible to compare QTLs with the mapped chromosomal location of previously known genes/QTLs. Most of the QTLs identified in this study were localized in adjacent regions with known QTLs that have previously been identified (Goudemand et al. 2013; Brown et al. 2015).

A QTLs on chromosome 1A (Qstb.iau-1) was isolate-specific for resistance to RM6 isolate from Iran. This QTLs did not align with any previously reported QTL or STB resistance genes, therefore we consume it as a potentially novel QTL. One isolate-specific QTL identified on chromosome 2A, co-located with previously known MQTL5 reported by Goudemand et al. (2013) for resistance under natural infection as also for two different isolates (IPO323 and IPO98099), but interestingly this QTL was not in association with IPO323 that used in our study. This can be concluded by the different nature of wheat germplasm and also the molecular markers that used in our study.

Two QTLs mapped on chromosomes 2B, Qstb.iau-3 was associated with resistance to two isolates (IPO323 and IPO86013) co-located with previously known QTLs reported by Goudemand et al. (2013) with the same isolate, IPO323. Another QTL, was isolate-specific for IPO98022, co-localized with previously known resistance gene (*Stb9*) and QTLs under natural field condition (Eriksen et al. 2003; Risser et al. 2011). Another QTL mapped on chromosomes 2D was in association with resistance to two Iranian *Z. tritici* isolates (RM6 and RM230) colocalized with previously reported meta-QTLs for resistance to septoria at seedling stage (Goudemand et al. 2013). As well as, identified QTL on chromosome 3B in this study for resistance to isolates IPO323 and IPO98022 was overlapped with previously reported meta-QTLs on this chromosome with the same isolates (Goudemand et al. 2013).

Three genomic regions were identified on chromosome 4A, of which two QTLs (Qstb.iau-8 and Qstb.iau9) were in association with resistance to Iranian *Z. tritici* isolates (RM22, Rm183 and RM230) overlapped with previously reported QTL on this region at adult stage (Goudemand et al. 2013), while another QTL, Qstb.iau-7 was in association with multiple isolates did not align with any previously reported QTLs or STB resistance genes, therefore we consume it as a potentially novel QTL.

Two QTLs on chromosomes 5A (*Qstb.iau-10*) and 5B (*Qstb.iau-12*) overlapped with known STB resistance genes, *Stb17* and *Stb1*, respectively. Two QTLs on chromosomes 5B (*Qstb.iau-11*) and 5D (*Qstb.iau-13*) were isolate-specific resistance and did not align with any previously reported QTLs or STB resistance genes, therefore we assume these are potentially novel QTLs. As far as we know, the new QTL on chromosome 5D for resistance to septoria in wheat is reported for the first time in this study. This region on chromosome 5D is also known as the introgressed region from *Aegilops umbellulata* and *Ae. tauschii* into wheat and is an important chromosomal location having resistance genes against leaf rust, stripe rust, soil-borne mosaic virus and powdery mildew (Bansal et al. 2020; Mohler et al. 2020; Liu et al. 2020; Fu et al. 2014). Although, the mapped QTLs on chromosomes 5A, 5B and 6A were overlapped with *Stb17*, *Stb1* and *Stb15* genes, respectively. This can confirm the results of the phenotyping experiment using the differential cultivars, that the high resistance pattern of genotypes that showed broad resistance to all isolates should be due to the presence of *Stb15*.

A QTL identified on chromosome 6B (*Qstb.iau-15*) overlapped with previously reported QTLs and Meta-QTLs for resistance to septoria under natural field infection at both seedling and adult stages (Eriksen et al. 2003; Goudemand et al. 2013). Novel genomic region (*Qstb.iau-16*) was identified on chromosome 6D, which was not aligned with any previously reported resistance genes or QTLs, therefore we assume it as a potentially novel isolate-specific resistance QTL. Three genomics regions associated with isolate specific resistance were co-localized with previously reported QTLs at adult stages (Goudemand et al. 2013).

To better understand the functional roles of QTL regions, significant MTAs were annotated and reviewed for putative candidate genes associated with diseases resistance in plant, as well as the expression analysis of these candidate genes also was examined. The results suggested that among 24 identified candidate genes, 10 genes showed expression under diseases stresses in different developmental stages of wheat. Overall, five genes belonged to protein kinase-like domain superfamily and a few genes belonged to leucine-rich repeat domain proteins exhibited a significantly higher expression under different diseases like as septoria, leaf rust and fusarium head blight (Supplementary Figure 3). These genes are well known as the most important plant gene, which has been involved in biotic stress response in different crop species (Yan et al. 2016; Andersen et al. 2018; Han, 2019; Pandian et al. 2020) as well as for resistance to STB in wheat (Louriki et al. 2021). Furthermore, we found several QTLs in close position of previously reported QTLs for resistance to *Z. tritici* isolates at both seedling and adult stages, which can be used as valuable sources for introgression of these QTLs into advanced wheat lines (Odilbekov et al. 2019). In addition to previously known QTLs, we found several potentially novel QTLs on chromosomes 1A, 4A, 5B, 5D and 6D. Detection of these putative new QTLs provided useful information that could be used to track favorable alleles for developing wheat cultivars resistant to STB. This knowledge can be used for generation of new allelic combination through cross between novel sources for resistance to STB (Riaz et al. 2020). As we used different isolates with diverse origins and identified potentially novel QTLs were associated with multiple isolates, this suggests that these QTLs remain effective as durable sources for resistance to STB, which can be used for future wheat breeding programs globally.

Conclusion

In this study, we investigated the resistance spectra of a diverse wheat global panel against ten *Z. tritici* isolates. Many of the Iranian cultivars and landraces were susceptible to most of the isolates. GWAS results revealed that 27 significant MTAs within 19 QTLs were significantly associated with resistance to different STB isolates. Although, most of the QTLs identified in this study co-localized with previously known STB QTLs/genes, five novel genomic regions associated with resistance to multiple *Z. tritici* isolates were identified. The QTL localized on chromosome 5D confers resistance to IPO86013 isolate, is the first report for resistance to STB in wheat on this chromosome. By aligning the significant MTAs against available wheat reference genome sequence and gene expression analysis, we characterized several candidate genes involved in plant defense mechanisms against pathogens. These genes are of interest and their exact roles in STB resistance remain to be functionally analyzed in the future. Molecular and functional characterization of these QTLs/genes eventually will enhance our understanding of how resistance is achieved and sheds light on biochemical mechanisms underlying resistance against STB. As well as, the significant QTLs and MTAs identified in this study will be further valued and can be used in marker-assisted selection for resistance to septoria in wheat breeding programs.

Declarations

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Tables

Table 1. List of isolates used for phenotypic analysis on 185 wheat genotypes at seedling stage under greenhouse conditions.

Isolates code	Origin	
	Country	Location
IPO02166	Iran	Dezful
IPO99031	France	Paris
IPO98022	France	Villaines la Gonais
IPO92034	Algeria	Guelma
IPO86013	Turkey	Adana
IPO323	Netherlands	W.Brabant
RM230	Iran	Bokan
RM22	Iran	Khozestan
RM6	Iran	Fars
RM183	Iran	Ardabil

Table 2. Summary of the linear mixed model (LMM) of percentages of leaf area with necrotic lesions bearing pycnidia of 10 isolates of *Zymoseptoria tritici* on 185 wheat genotypes

Genotype	7718.23	184	41.94	***
Isolate	1398.49	9	155.38	***
Genotype × Isolate	14317.59	1656	8.64	***

Table 3. Pearson correlation coefficient and broad-sense heritability (h2) for response to *Zymoseptoria tritici* isolates against 185 wheat genotypes across three replications under controlled greenhouseconditions.

Isolates	IPO02166	IPO99031	RM230	RM22	RM6	RM183	IPO98022	IPO92034	IPO86013	IPO323	Heritability
IPO02166	1										0.98
IPO99031	0.57**	1									0.97
RM230	0.28*	0.52*	1								0.97
RM22	0.49*	0.64**	0.63**	1							0.99
RM6	0.45*	0.55**	0.55**	0.80**	1						0.99
RM183	0.47*	0.58**	0.60**	0.84**	0.90**	1					0.97
IPO98022	0.37*	0.48*	0.54**	0.75**	0.81**	0.85**	1				0.92
IPO92034	0.26*	0.39*	0.45*	0.65**	0.66**	0.69**	0.80**	1			0.98
IPO86013	0.31*	0.42*	0.48*	0.70**	0.71**	0.75**	0.82**	0.82**	1		0.98
IPO323	0.29*	0.41*	0.48*	0.68**	0.73**	0.74**	0.81**	0.79**	0.90**	1	0.95

Table 4. Means of disease severity of wheat genotypes to different *Zymoseptoria tritici* isolates in three clusters

Cluster	No.Genotypes	IPO2166 IPO92034	IPO99031 IPO86013	RM230	RM22	RM6	RM183	IPO9802250	IPO323
1	63 38.44	56.61 40.61	43.10	44.24	39.15	38.06	39.92	35.79	30.98
2	85 51.61	66.57 50.68	60.78	53.89	55.49	61.12	59.91	54.32	45.14
3	37 8.22	43.59 9.08	30.39	25.77	15.03	13.34	13.16	9.28	7.76

Table 5. summary of the septoria tritici blotch resistance quantitative trait loci identified against 10 *Zymoseptoria tritici* isolates in the panel of 185 wheat genotypes.

QTL	Associated marker	Isolate	Chromosome	Physical position (bp)	Map Position (cM)	P value	MAF
<i>Qstb.jiau-11</i>	5332931	RM6	chr1A	2187418	7.5	6.08E-05	0.43
<i>Qstb.jiau-22</i>	4544165	IPO99031	chr2A	748128615	113.78	7.16E-05	0.49
	3533473	IPO99031	chr2A	776037491	120.68	3.43E-05	0.31
<i>Qstb.jiau-33</i>	3064517	IPO323	chr2B	11132	0.57	6.27E-05	0.17
	1864355	IPO323, IPO86013	chr2B	11066	1.26	3.60E-05	0.28
<i>Qstb.jiau-44</i>	1093912	IPO98022	chr2B	775155639	96.12	4.27E-05	0.48
<i>Qstb.jiau-55</i>	1265720	RM230	chr2D	12587173	11.54	4.99E-05	0.20
	3937084	RM6	chr2D	22776052	21.81	5.70E-05	0.48
<i>Qstb.jiau-66</i>	1205624	IPO98022	chr3B	310392089	53.24	3.49E-05	0.18
	1124803	IPO323	chr3B	557176126	65.26	6.95E-05	0.14
<i>Qstb.jiau-77</i>	5582113	IPO98022, IPO99031, RM6, RM183	chr4A	140700686	21.83	2.96E-05	0.11
<i>Qstb.jiau-88</i>	2257551	RM22, RM183	chr4A	641506835	96.35	9.54E-05	0.23
<i>Qstb.jiau-99</i>	1238557	RM230	chr4A	722708344	121.39	4.36E-05	0.33
<i>Qstb.jiau-100</i>	2258488	RM183	chr5A	568491318	80.07	5.20E-06	0.17
<i>Qstb.jiau-111</i>	5970385	IPO02166	chr5B	488112132	44.5	2.26E-05	0.09
	1088825	IPO02166	chr5B	490053583	45.23	3.16E-05	0.10
<i>Qstb.jiau-122</i>	2354562	IPO92034	chr5B	557353675	78.82	2.58E-05	0.19
<i>Qstb.jiau-133</i>	6038202	IPO86013	chr5D	541681037	137.5	1.207E-05	0.31
	7157166	IPO86013	chr5D	541902658	138.2	1.253E-05	0.35
<i>Qstb.jiau-144</i>	1110173	IPO92034	chr6A	5125140	8.31	7.85E-05	0.40
<i>Qstb.jiau-155</i>	1009838	IPO02166	chr6B	129858768	25.34	3.68E-05	0.39
	1266810	IPO98022	chr6B	648849374	37.82	8.80E-05	0.39
	5577074	IPO98022	chr6B	655271759	41.64	2.17E-05	0.43
<i>Qstb.jiau-156</i>	2275399	IPO98022	chr6D	433578091	72.21	6.16E-05	0.45
<i>Qstb.jiau-177</i>	4008741	IPO86013	chr7A	670929737	75.21	1.606E-05	0.30
<i>Qstb.jiau-188</i>	3020733	RM22	chr7D	15219082	11.07	9.15E-05	0.19
<i>Qstb.jiau-199</i>	2242097	IPO323	chr7D	556246143	119.31	6.99E-05	0.09

Table 6. Annotated candidate genes (CGs) associated with significant MTAs for resistance to septoria tritici blotch in a panel of 185 wheat genotypes.

Associated marker	Chr; Genomic Location	Genes in 5 Mb region (Genomic Location)	Protein domain name	Gene Ontology Annotation (GO IDs)		
				Biological Processes Function	Cellular Component	Molecular
5332931	1A; 2187418	TraesCS1A02G004400 (1A:2,642,460-2,648,016)	Homeobox-like domain superfamily	N/A	DNA binding (GO:0003677)	N/A
4544165	2A; 748128615	TraesCS2A02G529900 (2A: 748,078,167-748,081,023)	Cytochrome P450	oxidation-reduction process (GO:0055114)	monooxygenase activity (GO:0004497)	integral component of membrane (GO:0016021)
3533473	2A; 776037491	TraesCS2A02G582500 (2A: 774,848,949-774,856,166)	Protein kinase-like domain superfamily	protein phosphorylation (GO:0006468)	protein kinase activity (GO:0004672)	plasma membrane (GO:0005886)
3064517	2B; 11132	TraesCS2B02G001400 (2B: 1,171,680-1,174,401)	Leucine-rich repeat domain superfamily	protein phosphorylation (GO:0006468)	protein kinase activity (GO:0004672)	N/A
1864355	2B; 11066	TraesCS2B02G001600 (2B: 1,248,784-1,252,232)	Leucine-rich repeat domain superfamily	protein phosphorylation (GO:0006468)	protein kinase activity (GO:0004672)	integral component of membrane (GO:0016021)
1093912	2B; 775155639	TraesCS2B02G587400 (2B: 774,704,212-774,709,042)	P-loop containing nucleoside triphosphate hydrolase	N/A	ADP binding (GO:0043531)	N/A
1265720	2D; 12587173	TraesCS2D02G029700 (2D:12290063-12292008)	Cytochrome P450	oxidation-reduction process (GO:0055114)	monooxygenase activity (GO:0004497)	integral component of membrane (GO:0016021)
3937084	2D; 22776052	TraesCS2D02G056600 (2D:22508389-22511305)	Leucine-rich repeat domain superfamily	N/A	protein binding (GO:0005515)	N/A
1205624	3B; 310392089	TraesCS3B02G225800 (3B:312148349-312189729)	Protein kinase-like domain superfamily	protein phosphorylation (GO:0006468)	protein kinase activity (GO:0004672)	N/A
1124803	3B; 557176126	TraesCS3B02G348200 (3B: 557,089,459-557,099,768)	P-loop containing nucleoside triphosphate hydrolase	N/A	nucleotide binding (GO:0000166)	N/A
5582113	4A; 140700686	TraesCS4A02G111900 (4A:136799266-136802539)	Protein kinase-like domain superfamily	protein phosphorylation (GO:0006468)	protein kinase activity (GO:0004672)	N/A
2257551	4A;641506835	TraesCS4A02G370600 (4A:642284232-642290461)	P-loop containing nucleoside triphosphate hydrolase	transmembrane transport (GO:0055085)	ATP binding (GO:0005524)	integral component of membrane (GO:0016021)
1238557	4A; 722708344	TraesCS4A02G458200 (4A:723513178-723518607)	Leucine-rich repeat domain superfamily (Virus-X resistance protein)	N/A	protein binding (GO:0005515)	N/A
2258488	5A; 568491318	TraesCS5A02G367700 (5A:567914382-567927397)	Protein kinase-like domain superfamily (Serine/threonine-protein kinase)	protein phosphorylation (GO:0006468)	protein kinase activity (GO:0004672)	N/A
5970385	5B; 488112132	TraesCS5B02G304000 (5B:488114868-488120798)	Leucine-rich repeat domain superfamily	N/A	protein binding (GO:0005515)	N/A
1088825	5B; 490053583	TraesCS5B02G304900 (5B: 488,820,167-488,826,003)	Leucine-rich repeat domain superfamily	N/A	ADP binding (GO:0043531)	N/A
2354562	5B; 557353675	TraesCS5B02G380400 (5B:558341804-558347101)	Protein kinase-like domain superfamily (Serine/threonine-tyrosine-protein kinase)	protein phosphorylation (GO:0006468)	protein kinase activity (GO:0004672)	N/A
6038202	5D; 541681037	TraesCS5D02G517500 (5D:539453646-	Leucine-rich repeat-containing N-terminal	N/A	protein binding (GO:0005515)	N/A

Table 6. Continued

Associated marker	Chr; Genomic Location	Genes in 5 Mb region (Genomic Location)	Protein domain name	Gene Ontology Annotation (GO IDs)		
				Biological Processes Function	Cellular Component	Molecular
1110173	6A; 5125140	TraesCS6A02G011000 (6A:5134588-5135865)	F-box-like domain superfamily	N/A	protein binding (GO:0005515)	N/A
1009838	6B; 129858768	TraesCS6B02G133000 (6B:129853633-129857011)	Leucine-rich repeat domain superfamily	protein phosphorylation (GO:0006468)	protein kinase activity (GO:0004672)	integral component of membrane (GO:0016021)
2275399	6D; 433578091	TraesCS6D02G327400 (6D:432719014-432725845)	Protein kinase-like domain superfamily	protein phosphorylation (GO:0006468)	protein kinase activity (GO:0004672)	N/A
4008741	7A; 670929737	TraesCS7A02G476700 (7A: 670,821,522-670,825,200)	Leucine-rich repeat domain superfamily	protein phosphorylation (GO:0006468)	protein kinase activity (GO:0004672)	integral component of membrane (GO:0016021)
3020733	7D; 15219082	TraesCS7D02G029200 (7D:14586729-14589615)	Protein kinase-like domain superfamily	protein phosphorylation (GO:0006468)	protein kinase activity (GO:0004672)	integral component of membrane (GO:0016021)
2242097	7D; 556246143	TraesCS7D02G435700 (7D:554835233-554837528)	Protein kinase-like domain superfamily	protein phosphorylation (GO:0006468)	protein kinase activity (GO:0004672)	N/A

Figures

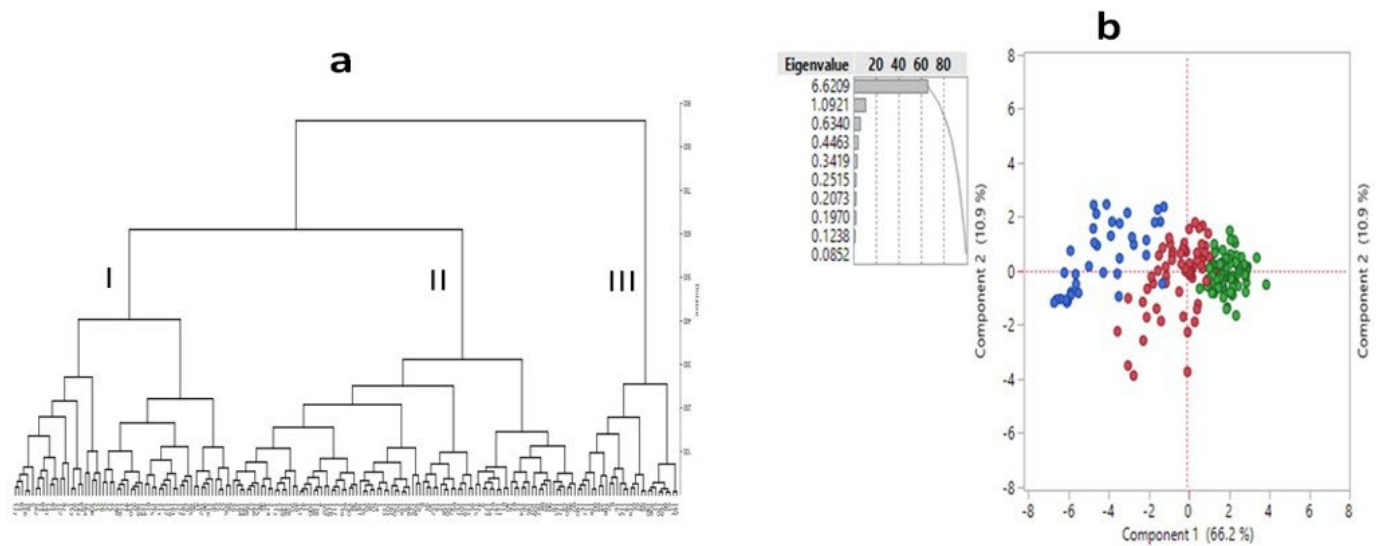


Figure 1

Cluster analysis (a) and principal component analysis (PCA) of 185 wheat genotypes based on mean disease severity data against ten *Zymoseptoria tritici* isolates.

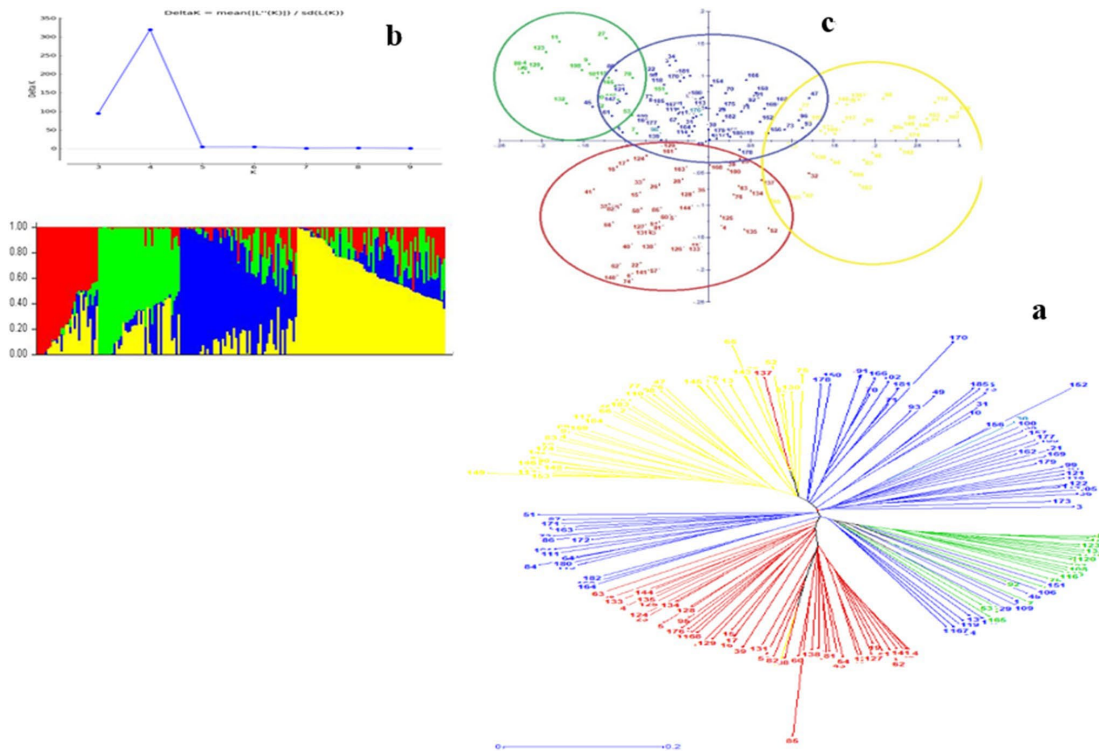


Figure 2

The neighbor-joining cluster analysis based on genetic dissimilarity in 185 wheat genotypes (a). The color of branches indicates accessions corresponding to the subpopulations in population structure analysis, Determination of the optimal value of K and population structure analysis (b) and principal coordinate analysis (c) of 185 wheat genotypes based on DArTseq markers.

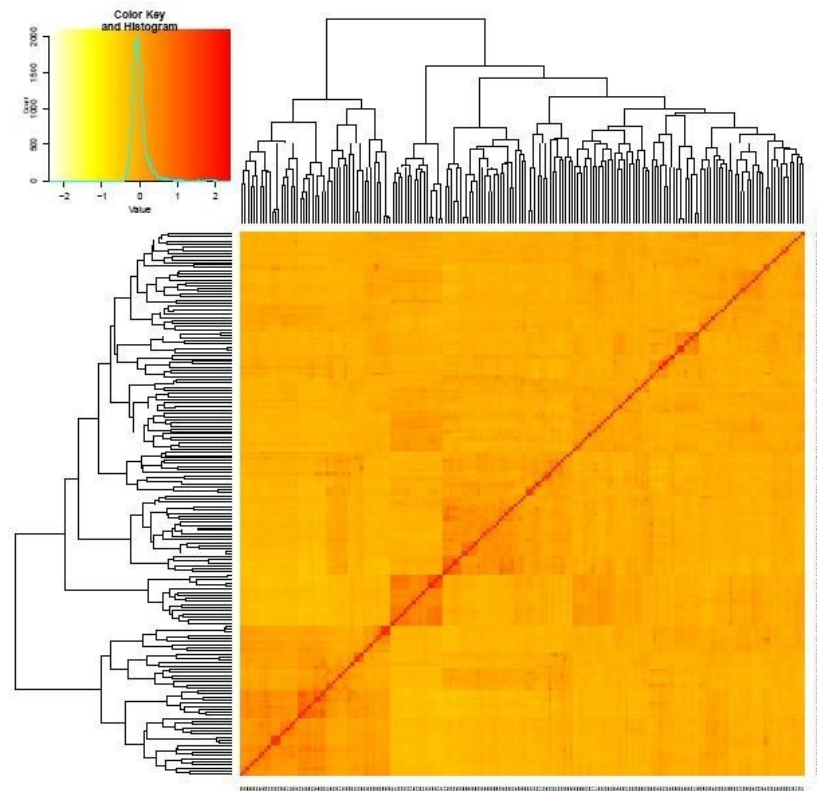


Figure 3

Heatmap plot of kinship matrix displaying relationships of 185 wheat genotypes based on DArTseq markers.

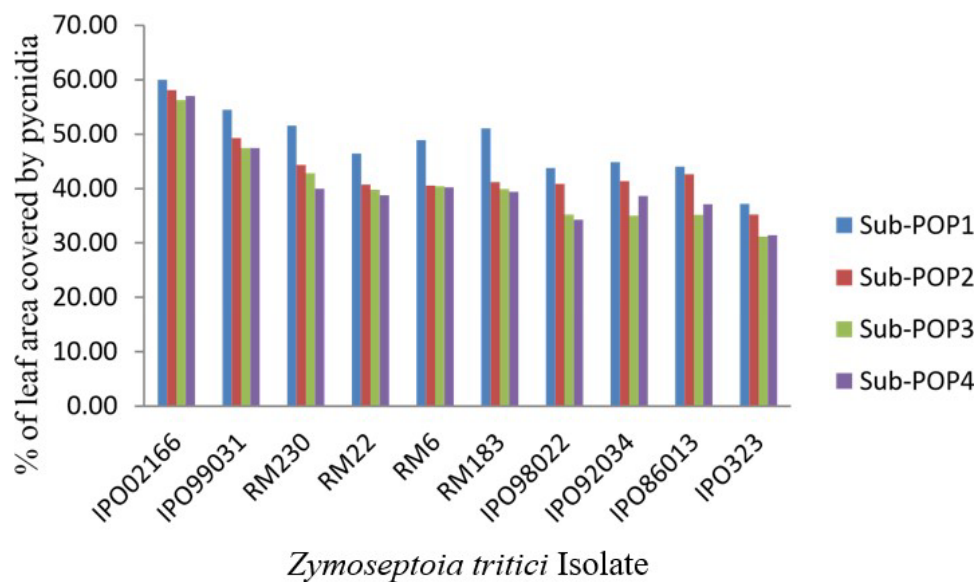


Figure 4

Different sub-population of 185 wheat genotypes panel that showed a different level of septoria tritici blotch disease severity (measured using the percentage of leaf area covered by pycnidia)

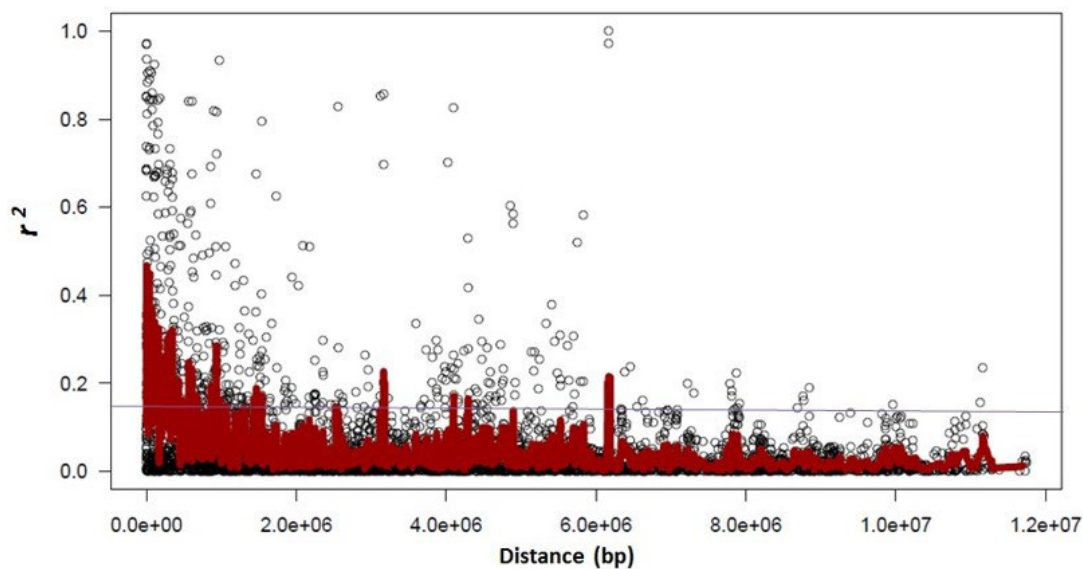


Figure 5

Linkage disequilibrium (LD) measured r^2 plotted vs. the physical map (bp) between pairs of DArTseq markers in a panel of 185 wheat genotypes

Figure 6

Circular Manhattan plot for association analysis between DArTseq markers and ten different *Z. tritici* isolates in 185 hexaploid wheat genotypes.

Supplementary Files

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- [SupplementaryFigure3.tiff](#)
- [SupplementaryTableS12.xlsx](#)
- [SupplementaryTableS21.xlsx](#)