

Article

Genetic Variability of Wheat Collection Selected with Respect to the Resistance to Leaf and Stem Rusts

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Abstract

Wheat (*Triticum aestivum* L.) is an economically important cereal crop. Its genetic diversity is a prerequisite for successful breeding for resistance to leaf and stem rust, as well as for other important traits. Therefore, the main objective of the study was to determine the level of genetic diversity of a file of 185 wheat accessions chosen to represent a wide range of resistance levels to leaf and stem rusts. They were evaluated at two locations in three years (2023–2025) for leaf and stem rust resistance by means of visual evaluation and fungus content using real-time PCR. BLUP values were then computed from these multi-environmental data. In addition, data on other traits such as heading time, plant height, and grain quality were collected. In the PCA, grain quality variables represented the first factor, explaining 41% of the total variability. Rust resistance was mainly associated with the second and the third factor. Population analysis revealed primary two ($K = 2$) and secondary six ($K = 6$) clusters, computed by using 13,274 DArT markers. The correlation between genotype and phenotype data was high ($R = 0.89$) when all traits were analyzed, and lower ($R = 0.58$) when only leaf and stem rust resistance variables were considered. Genetic diversity was also studied through an association analysis between markers and phenotypic traits using GLMs and MLMs. As a result, 300 statistically significant marker-trait associations (MTAs) were identified ($p \leq 3.77 \times 10^{-6}$). Of these MTAs, 180 were associated with rust resistance variables and 120 with other traits. A BLAST search of rust resistance MTAs identified 128 putative genes; 47 of them can be taken for candidate genes related to fungal resistance. The results show that association analysis can help to explain the genetic diversity of selected wheat accessions, and that leaf and stem rust resistance are complex traits involving many non-specific resistance genes.

Keywords: wheat diversity; leaf rust; stem rust; grain quality; DArT markers; genotype-phenotype correlation; GWAS; candidate genes



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1. Introduction

Bread wheat (*Triticum aestivum* L.) is a self-pollinated species. Wheat is an allo-hexaploid ($2n = 42$) containing three distinct but related genomes, A, B, and D, each with seven chromosomes. It has a large genome of 16×10^9 bp, more than 80% of which consists of repetitive DNA [1]. The genetic diversity of wheat varieties is the basis for successful breeding. It has been evaluated using several methods, including Restriction Fragment Length Polymorphism (RFLP), Randomly Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP), and Simple Sequence Repeats (SSRs).

Recently, Diversity Arrays Technology (DArT) was developed as a high-throughput method of the whole-genome genotyping [2]. DArT is, among others, widely used in diversity studies [3] and in genome-wide association studies (GWASs) [4,5]. GWAS use single-nucleotide polymorphism markers (SNPs) that are dispersed throughout the genome to identify associations with agronomically important traits.

The main objectives of wheat breeding remained largely similar over the years: increasing yield potential, improving resistance to diseases, enhancing tolerance to abiotic stresses, and improving grain quality. All these traits are quantitatively inherited and are controlled by multiple genes. Moreover, they are affected by environmental factors. Although it is necessary to know information about the values of several traits in a selected set of varieties at once, publications that assess variability in such a comprehensive manner are rare.

To maximize wheat production, optimizing several agronomic traits such as, for example, yield, quality parameters, and disease resistance is essential. This study is focused on rusts, namely leaf rust (*Puccinia triticina*), and stem rust (*P. graminis* f. sp. *tritici*), which are among the most damaging diseases that affect wheat. Fungicides are commonly used to prevent yield losses, but the most economical, efficient, and environmentally friendly strategy is the use of resistant varieties. Two types of resistance strategies may involve constitutive and induced defence mechanisms. Thus far, 83 stem rust (Sr), 130 stripe rust (Yr), and 117 leaf rust (Lr) resistance genes have been identified [5]. Resistance to wheat rusts can be classified into race-specific resistance, which is manifested mainly at the seedling stage, and race-non-specific resistance, which is expressed in later phenological phases and controlled by minor genes or QTLs. This so-called adult plant resistance is more durable because it does not create extensive selection pressure on fungal populations. However, these genes or QTLs are often difficult to detect due to their small effects [6].

Today, GWAS is widely used to find new resistance genes or QTLs for rust diseases. To be successful, this approach requires a diversity panel consisting of accessions with sufficient uniformity in other traits [7]. However, identified marker trait associations (MTAs) are often difficult to validate and use in breeding programmes [8]. It is now generally accepted that over the last several decades, the breeding process has caused a significant reduction in diversity [9]. Focusing on the trait of interest may reduce attention to variability in other traits. Selective breeding may thus contribute to genetic erosion. However, breeding for several traits at the same time is difficult; more genetic variance is preserved in the selected progenies than when each trait is bred for separately [10].

Therefore, the main objective of this study was to determine the level of genetic diversity of a file of wheat accessions chosen to represent a wide range of resistance levels to leaf and stem rusts. In addition to leaf and stem rust disease evaluations, several other phenotypic traits, including heading time and plant height, and grain quality parameters, such as gluten content, starch content, hardness, Zeleny test results, and nitrogen content, were collected and compared with the results of genotyping performed using Diversity Arrays Technology. Two hypotheses were tested: (1) there is no variability within the file of selected wheat accessions; (2) in the case of an alternative hypothesis, it is not possible to explain the variability by phenotypic traits, especially by grain quality traits. If the second hypothesis is refused, it will be possible to identify loci related to the tested traits.

2. Materials and Methods

2.1. Plant Material

In total, 185 bread wheat accessions were used in this study, of which 150 were wheat varieties and 35 were breeding lines. Spring wheat was represented by 62 genotypes and winter wheat by 123 accessions. They were selected by breeders based on twenty years of

experience to cover a wide range of resistance levels to leaf rust and stem rust. All material was obtained from the SELGEN breeding station, Sibrina, Czech Republic.

2.2. Phenotyping

The field experiments were conducted during the 2023–2025 growing seasons at the experimental field of Selgen and Prague-Ruzyně according to the optimized protocol [11].

Leaf rust (Pt) and stem rust (Pg) were chosen as model pathogens. Each disease was evaluated at a separate field location within the experimental field of Selgen and Prague-Ruzyne. In all trials, accessions were grown in three-row plots arranged in two randomized replications. Individual rows were separated by a spreader row consisting of a mixture of susceptible varieties (Michigan Amber, Baletka, and Victo). Control varieties with well-characterized resistance and susceptibility, namely Carrol and Skif (resistant controls), and Samanta and Michigan Amber (susceptible controls), were included. The spatial arrangement of genotypes within the field trials was randomized.

The inoculum consisted of a mixture of urediniospores representing different races with defined virulence/avirulence profiles. The source of infectious material was wheat leaves infected with leaf rust or stem rust collected throughout the Czech Republic during the previous growing season. The inoculum was multiplied on seedling plants under greenhouse conditions. Artificial inoculation in the field was carried out at growth stages BBCH 29–37 using a mixture of isolates of either stem rust or leaf rust applied to the spreader rows by spraying an aqueous suspension of urediniospores on the leaves. To enhance infection pressure, varieties at growth stages BBCH 31–37 were additionally inoculated by stem injection with a mixture of either stem rust or leaf rust isolates. This method is particularly suitable when long-term temperature conditions are suboptimal for foliar inoculation. The timing of artificial inoculation differed between rust species due to their distinct temperature requirements for successful infection.

Disease severity was assessed on multiple dates according to disease development. A standard 1–9 rating scale used by the Central Institute for Supervising and Testing in Agriculture (CISTA), Czech Republic, was applied (1 = highly susceptible; 9 = highly resistant). The highest value for each locality and year and the resulting matrix (Table S1) were used in the subsequent analyses.

Resistance to stem and leaf rusts was also assessed by quantifying pathogen content in host plant tissue using real-time PCR. For this purpose, primers and TaqMan probes specific to *P. triticina* and *P. graminis*, and *T. aestivum* were designed using the sequences of β -tubulin of *Puccinia* spp. and α -tubulin for wheat (Table 1). Specificity was tested on a panel of wheat fungal pathogens, and the reaction efficiency and collinearity between pathogen and host plant Ct values were established—for Pg: $E = 0.88$, $R^2 = 0.99$; for Pt: $E = 0.94$, $R^2 = 1.00$; for wheat: $E = 0.90$, $R^2 = 1.00$. Five plants were randomly collected across the entire row plot at the time of the final resistance evaluation at both localities. DNA was extracted from leaves and stems using CTAB according to an optimized protocol. PCRs were run in a duplex arrangement in triplicate on ABI PRISM 7900 (Applied Biosystems, Foster City, CA, USA) or QuantStudio 6 (ThermoFisher Scientific, Waltham, MA, USA). The reaction mixture of 20 μ L volume consisted of 1 $\times$ TaqMan Universal Mastermix II (ThermoFisher Scientific, Waltham, MA, USA), 0.25 μ M of Pg/Pt and Ta primer pairs, 0.25 μ M of Pg/Pt and Ta TaqMan probes, and 200 ng of DNA. The reaction consisted of 10 min at 95 $^{\circ}$ C and 40 cycles of 95 $^{\circ}$ C for 15 s and 60 $^{\circ}$ C for 1 min. The inverse ratio of the infestation rate, i.e., the ratio of the Ct value of the fungus to the Ct value of the host plant, was used in subsequent analyses (Table S1).

Table 1. Primer and probe sequences.

	Forward	Reverse	Probe
Pg	TTTCCCTCGATTGCACTTCTTC	GCTTCCACGAGCGGTAAGC	6FAM-TGGTCGGATTTCGCTC
Pt	CCCCCGATTGCATTCTTC	TCCACGGGCGGTAAGT	6FAM-TGGTCGGATTTCGCTC
Ta	CCCGCCACGGCAAGTAC	ACATCCTTGGGCACAACATCA	VIC-TGCTGCCTCATGTACCGT

Pg is *Puccinia graminis*; Pt is *P. tritricina*; Ta is *Triticum aestivum*.

For phenotyping during the vegetation period and subsequent evaluation of grain quality parameters, plants were grown in field experiments on plots of 2 m². Fertilization for winter trials consisted of 76 kg ha^{−1} K₂O, 82.4 kg ha^{−1} P₂O₅, and 108 kg ha^{−1} N, applied in two doses at the end of February and in BBCH 25–30. For spring wheat trials, 87 kg ha^{−1} K₂O, 85 kg ha^{−1} P₂O₅, and 69 kg ha^{−1} N in two doses were applied in BBCH 25–30 and at the turn of April and May. Protection against weeds and animal pests was carried out according to plant protection methodologies. Growth regulator and fungicide protection were not applied. The following field parameters were recorded: heading time, defined as the day when at least 75% of the plants had reached BBCH 51, and plant height. Plots were harvested with a combine harvester. Samples were processed using an Infratec 1241 (Foss, Hilleroed, Denmark) instrument. Grain quality parameters, such as wet gluten content, starch content, hardness, Zeleny test, and nitrogen content, were analyzed using NIR spectroscopy, and data were collected for further analyses.

2.3. Genotyping

Approximately 30 plants per wheat accession at the seedling stage were pooled, and genomic DNA was extracted using the CTAB method according to an optimized protocol. DNA quality and quantity were assessed electrophoretically and using a Qubit 4 fluorometer (Invitrogen, Waltham, MA, USA). Samples were genotyped using DArT analysis at the University of Canberra, Canberra, Australia.

DArTseq markers were mapped using two references: Wheat Chinese Spring v. 2.1 and Wheat consensus map v. 4 (www.diversityarrays.com), developed by DArT Pty. Ltd., Canberra, Australia. A total of 103,307 DArT markers were scored. After removing markers with ≥10% missing data, and those with a frequency ≤ 0.05, 13,274 markers were retained for further analysis. Therefore, the final data matrix consisted of 185 genotypes and 13,274 markers.

2.4. Data Analysis

Population structure analysis was performed using Bayesian statistics implemented in Structure version 2.3.4 [12]. Ten independent runs for 1–10 groups ($K = 1–10$) were performed using a locprior model with admixture and correlated allele frequencies [13,14], with 50,000 Markov chain iterations after a burn-in period of 10,000 iterations. The optimal value of K was estimated based on $\ln(K)$ and the ΔK calculation, which considers the rate of change in the $\ln P(D)$ values among successive K runs to account for patterns of dispersal that are not homogeneous among populations [15]. The posterior probability (Pr) ($X|K$) with which the sample data (X) fitted K clusters was used in the following analyses.

Data for the phenotypic traits (heading time, plant height, and grain quality parameters, such as gluten content, starch content, hardness, Zeleny test, and nitrogen content) were collected and the percentage data (nitrogen content) were arcsin transformed. Effects were tested using general linear models and ANOVA implemented in Statistica v. 14.0.0.15 (TIBCO Software Inc., San Ramon, CA, USA), with the phenotypic data as the response variable, and genotype, locality and year of the field experiment as factors. Genotype was treated as fixed effect and locality and year as random effects. Model included genotype $\times$ environment interactions. In addition, Principal Component Analysis (PCA) of all collected traits, and Canonical Correlation Analysis (CCA) were calculated in Statistica software.

CCA was computed between phenotype data and the posterior probability (Pr) ($X|K$) with which the sample data (X) fitted K clusters.

Best linear unbiased predictions (BLUPs) for cultivar performance were estimated using ASReml-R v. 4.1.0.149 (VSN International, Hemel Hempstead, UK) based on raw phenotypic data for four traits: visual assessment of resistance to leaf and stem rust, and quantitative determination of leaf and stem rust in wheat leaf and stem tissues. For each variable, the following linear mixed model was fitted:

$$y_{ijk} = \mu + G_i + Y_j + L_k + (G \times L)_{ik} + (G \times Y)_{ij} + \varepsilon_{ijk},$$

where y_{ijk} is the phenotypic observation of the i th cultivar in the j th year and k th locality; μ is the overall mean (fixed intercept); G_i is the random effect of cultivar (genotype); Y_j is the random effect of year; L_k is the random effect of locality; $(G \times L)_{ik}$ is the random cultivar $\times$ locality interaction; $(G \times Y)_{ij}$ is the random cultivar $\times$ year interaction; and ε_{ijk} is the residual error term.

Broad-sense heritability (H^2) of genotype means across environments was calculated based on the estimated variance components from the fitted mixed model as follows:

$$H^2 = \frac{\sigma_G^2}{\sigma_G^2 + \frac{\sigma_{GE}^2}{e} + \frac{\sigma_e^2}{e}},$$

where σ_G^2 is the genetic variance (cultivar effect), σ_{GE}^2 is the total genotype $\times$ environment interaction variance (sum of cultivar $\times$ locality and cultivar $\times$ year components), σ_e^2 is the residual variance, and e is the number of environments (year $\times$ locality combinations).

The pairwise linkage disequilibrium (LD) between the markers, based on their correlations (r^2), was calculated using the Java-based open-source software TASSEL v. 5.2.93 [16]. TASSEL was also used to compute latent variables for multidimensional scaling analysis (MDS). A scatterplot was constructed in Statistica software.

To detect significant associations, the combined MLM and GLMs was applied using TASSEL software v. 5.2.93 [16]. To confirm the correction due to K and Q matrices, the distribution lines in each quantile–quantile (QQ) analysed. Significant marker-trait associations (MTAs) were selected based on the Bonferroni-corrected threshold of $\alpha = 0.05/n$, where n is the number of markers [17]. Blast analysis using blastn algorithm of selected loci was performed against standard databases (NCBI, EMBL, DDBJ).

3. Results

3.1. Phenotypic Variation

A wheat panel consisting of 185 genotypes was selected to reflect the widest possible range of responses to leaf and stem rusts. Moreover, four variables related to resistance (visual evaluation of Pg, visual evaluation of Pt, Pg content, and Pt content) were established, and data were collected at two localities and over three years (Table S1). The preliminary phenotypic results confirmed the expected diversity. Although the mean visual evaluation scores of Pg and Pt were similar (5.1 and 5.2, for Pg and Pt, respectively), the variance was higher for Pg (3.24) than for Pt (0.49). The upper limit was the same for Pg and Pt (7.8), but the lower limit differed between Pg and Pt (1.3 and 3.2, respectively). The situation was similar for Pg content and Pt content (Table 2). These results indicate that, while the resistance to leaf rust was mostly of moderate level, the response to stem rust was more diversified.

Table 2. Descriptive statistics of traits evaluated within the study.

Variable	Mean	Min	Max	SD	Median
Pg evaluation	5.1	1.3	7.8	1.8	5.8
Pt evaluation	5.2	3.2	7.8	0.7	5.2
Pg content	1.22	1.01	1.48	0.10	1.23
Pt content	1.06	0.96	1.41	0.06	1.05
Heading	144.1	125.7	159	6.3	144.7
Plant height	89.7	55.3	116.7	10.6	90
N-compound content	0.13	0.11	0.18	0.01	0.13
Zeleny test	41.8	24.8	72.9	8.7	40.5
Hardness	46.2	−0.6	77.3	10.6	47.4
Starch content	68.4	60.8	72.8	2.2	68.7
Wet gluten content	29.2	19.9	44.6	5.7	27.7

Data on the resistance of wheat accessions to leaf and stem rusts collected at two localities and over three years were analyzed using ANOVA. All factors, namely year, locality, and wheat genotype, as well as the year $\times$ locality interaction, were statistically significant for all four variables (Table 3, Figure 1). The impact of wheat genotype was the third most significant factor, after year and locality.

Table 3. ANOVA table of four variables.

Pg Evaluation	SS	df	MS	F	p
Intercept	36,082.41	1	36,082.41	45,008.01	0.000
Year	193.74	2	96.87	120.83	0.000
Locality	4.47	1	4.47	5.58	0.019
Genotype	4755.61	185	25.71	32.06	0.000
Year $\times$ Locality	98.98	2	49.49	61.73	0.000
Year $\times$ Genotype	416.13	370	1.12	1.40	0.001
Locality $\times$ Genotype	508.07	185	2.75	3.43	0.000
Year $\times$ Locality $\times$ Genotype		0			
Error	291.81	364	0.80		
Pt Evaluation	SS	df	MS	F	p
Intercept	26,332.62	1	26,332.62	18,967.22	0.000
Year	42.81	2	21.41	15.42	0.000
Locality	78.28	1	78.28	56.38	0.000
Genotype	1208.60	185	6.53	4.71	0.000
Year $\times$ Locality	80.97	2	40.49	29.16	0.000
Year $\times$ Genotype		0			
Locality $\times$ Genotype		0			
Year $\times$ Locality $\times$ Genotype		0			
Error	1127.32	812	1.39		
Pg Content	SS	df	MS	F	p
Intercept	1574.942	1	1574.942	109,224.2	0.000
Year	4.036	2	2.018	139.9	0.000
Locality	4.863	1	4.863	337.3	0.000
Genotype	18.780	185	0.102	7.0	0.000
Year $\times$ Locality	5.307	2	2.653	184.0	0.000
Year $\times$ Genotype	6.749	370	0.018	1.3	0.013
Locality $\times$ Genotype	3.811	185	0.021	1.4	0.002
Year $\times$ Locality $\times$ Genotype		0			
Error	5.076	352	0.014		
Pt Content	SS	df	MS	F	p
Intercept	1006.978	1	1006.978	39,872.91	0.000
Year	3.205	2	1.603	63.46	0.000
Locality	0.952	1	0.952	37.70	0.000
Genotype	11.077	185	0.060	2.37	0.000
Year $\times$ Locality	1.378	2	0.689	27.28	0.000
Year $\times$ Genotype	8.542	370	0.023	0.91	0.795
Locality $\times$ Genotype	4.321	185	0.023	0.92	0.719
Year $\times$ Locality $\times$ Genotype		0			
Error	7.677	304	0.025		

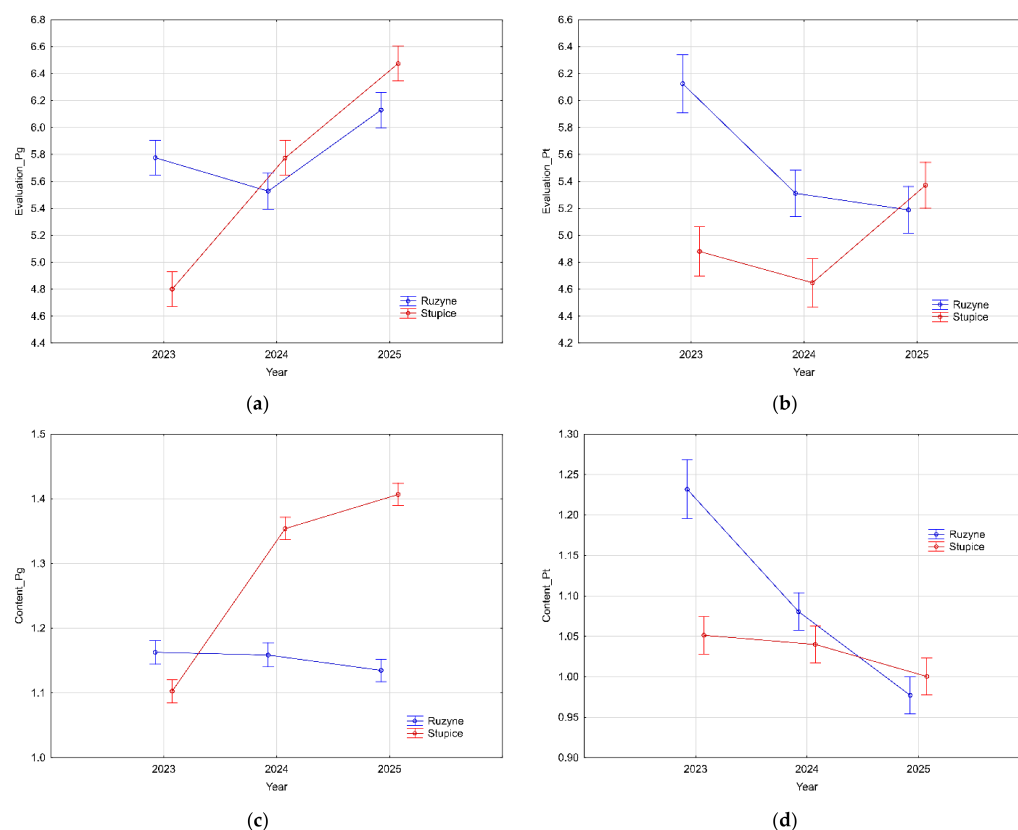


Figure 1. ANOVA plot of variables: (a) Pg evaluation; (b) Pt evaluation; (c) Pg content; (d) Pt content collected over three years in two localities; y-axis represents mean values of variables; vertical bars denote 0.95 confidence intervals.

In addition to resistance to rust diseases, other phenotypic data were collected in one locality over three years (Table S1).

Principal Component Analysis was performed to assess the relationship among all variables. The first three factors explained 69.6% of the total variability present in the data set. The first factor (40.6% of the variability) was positively associated with the Zeleny test, nitrogen content, wet gluten content, and negatively associated with starch content. The second factor (16.1% of the variability) was represented by Pg evaluation and Pg content, and the third factor (12.9% of the variability) was represented by Pt evaluation and Pt content (Figure 2). Although the panel of wheat accessions was chosen to represent as much variability in the resistance to stem and leaf rusts as possible, grain quality parameters showed a higher level of diversity within the dataset.

3.2. Genetic Diversity and Population Structure

Genotyping was performed using DArT analysis. After quality filtering, 13,274 DArTs were selected, 11,172 of which were mapped to the wheat chromosomes of the Chinese Spring v. 2.1 reference (Table S2). Most of the markers were located in the B genome (4489), followed by the A (4006) and D genomes (2677). The number of markers per chromosome ranged from 164 (chromosome 4D) to 866 (chromosome 2B). The greatest proportion (34%) of polymorphic, anchored markers resided in the B sub-genome. The average polymorphic information content (PIC) value was 0.24 (Table 4).

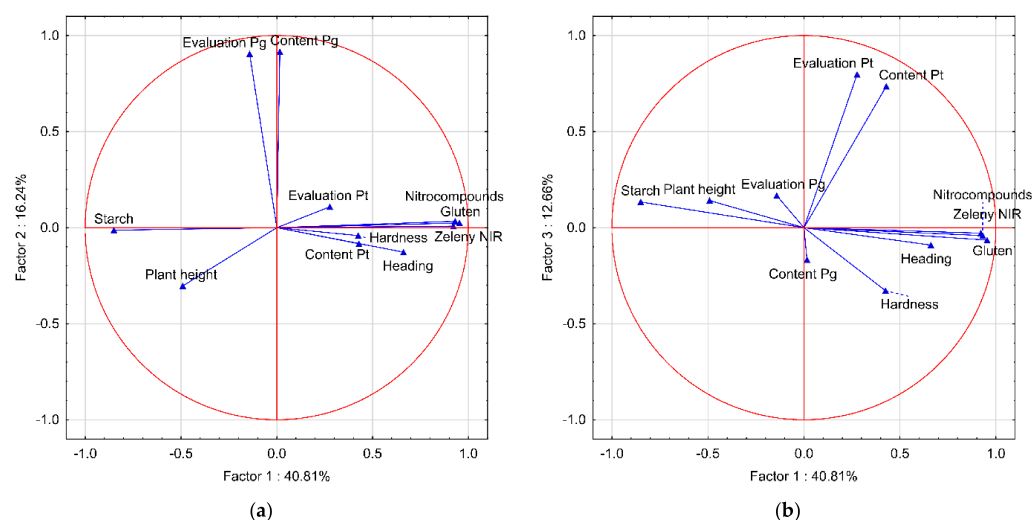


Figure 2. PCA loading plots showing the projection of variables onto the PC1–PC2 (a) and PC1–PC3 (b) planes. Arrows represent individual traits, with their direction and length indicating the strength and direction of their correlation with the principal components. The red circle represents the unit correlation circle. Percentages indicate the proportion of variance explained by each component. Traits located close to each other are positively correlated, while traits oriented in opposite directions are negatively correlated.

Table 4. Distribution of SNPs on wheat chromosomes mapped in Chinese Spring 21.

Chromosome	n (SNPs)	PIC	SD
1A	500	0.23	0.15
2A	633	0.25	0.14
3A	566	0.23	0.14
4A	367	0.23	0.13
5A	646	0.27	0.15
6A	497	0.25	0.15
7A	797	0.25	0.14
1B	695	0.20	0.13
2B	866	0.26	0.15
3B	767	0.25	0.14
4B	307	0.27	0.15
5B	607	0.28	0.14
6B	616	0.24	0.14
7B	631	0.23	0.11
1D	397	0.23	0.14
2D	576	0.25	0.13
3D	376	0.18	0.11
4D	164	0.19	0.11
5D	333	0.22	0.12
6D	344	0.22	0.13
7D	487	0.18	0.11
Unknown	2102	0.22	0.13
Total	13,274	0.24	0.03

PIC—polymorphic information content; SD—standard deviation of PIC values. Chromosomes are grouped by genomes A, B, and D. “Unknown” includes SNP markers with unresolved chromosomal positions.

Analysis of population structure showed ΔK peaks at $K = 2$, and $K = 6$, supporting two, and subsequently six, as the most probable number of subpopulations. Multidimensional scaling revealed two groups among wheat accessions, with some individuals positioned between them (Figure 3). The two subpopulations and groups in Figure 4a represent the

spring and winter type of wheat accessions. For $K = 6$, the value of α was 0.20, which means that most wheat accessions were admixed. According to the highest Q value, each wheat accession was assigned to a K subpopulation (Figure 3b, Table S4). While $K2$ and $K5$ were predominant in winter wheat accessions (88.7% and 93.8%, respectively), $K3$ and $K4$ occurred mainly in spring wheat genotypes (80.1% and 91.8%, respectively). $K2$ had the highest representation (44.4%). Subpopulations with the highest F_{st} values were $K4$ and $K6$ (0.68 and 0.67, respectively). Typical representatives for each K with a Q value higher than 90% were as follows: for $K2$ cv. Biscay from Germany, Julie from the Czech Republic, and line SG-S2174-21 (CZE); for $K3$ cv. Daphne (CZE), SW Kadrilj from Sweden; for $K4$ Anabel (CZE), Saxana (CZE), Tercie (CZE); for $K5$ cv. Tobak from Germany; for $K6$ cv. Complet from Germany, Samanta (CZE). The highest value of Q in $K1$ was found in cv. Solindo from France (79%).

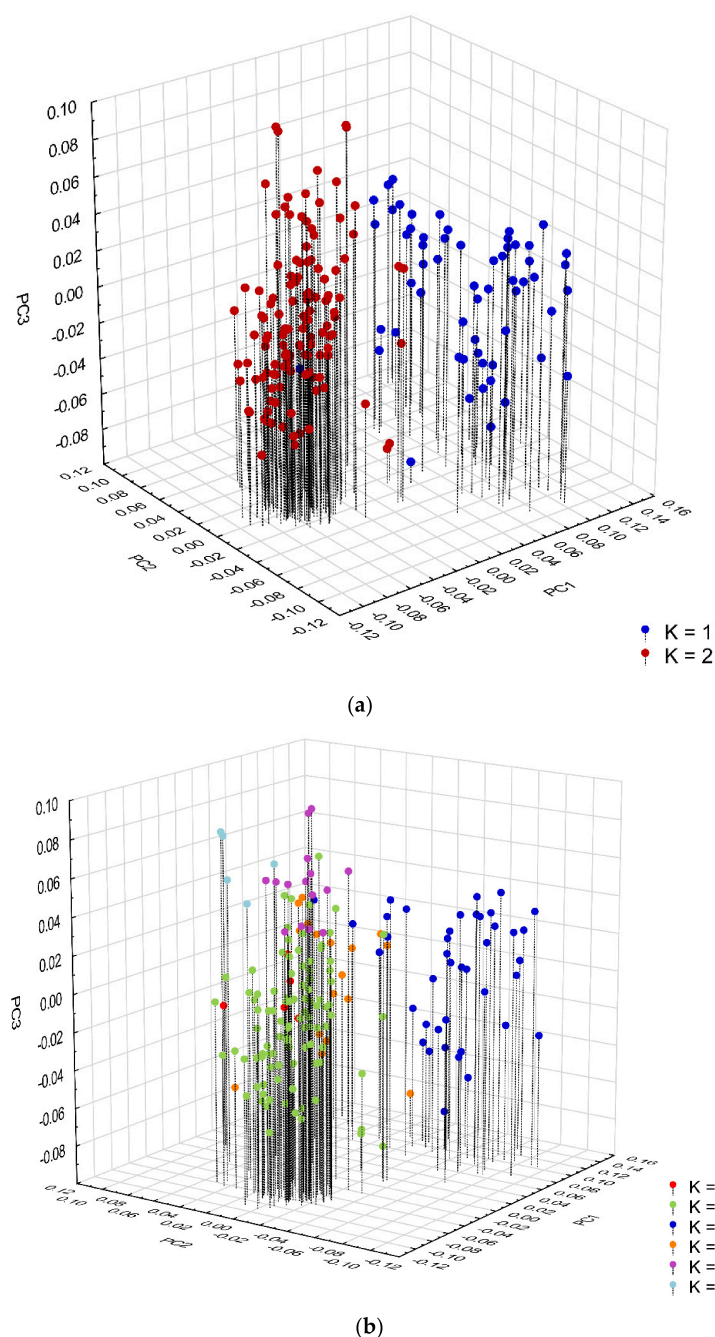


Figure 3. Three-dimensional MDS plot with the number of subpopulation $K = 2$ (a), and $K = 6$ (b).

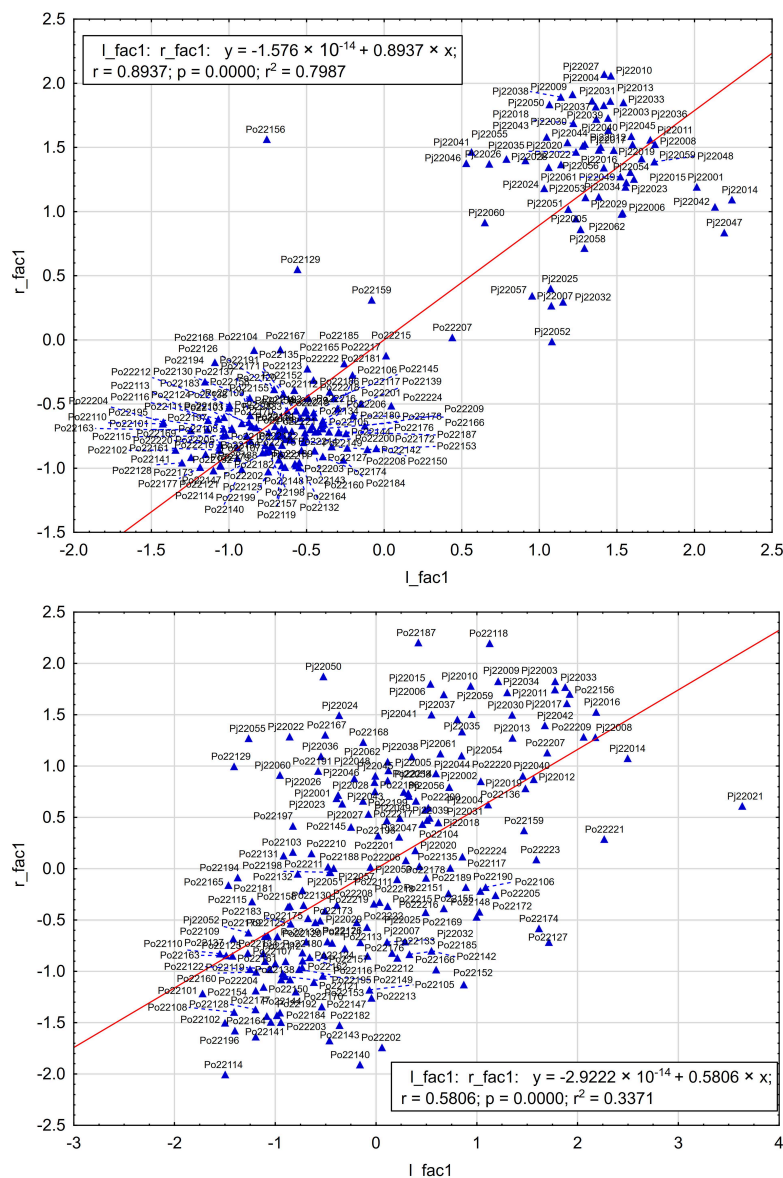


Figure 4. Correlation between genotypic and phenotypic data based on **(top)** all variables; **(bottom)** four rust resistance-related variables; x-axis (l_fac1) represents the first canonical variable computed from Q values of K6 populations; y-axis (r_fac1) represents the first canonical variable computed from phenotype data.

3.3. Correlation Between Genotype and Phenotype

A high level of correlation was found between the genotype and phenotype data ($R = 0.89$; $p < 0.01$). For the phenotypic data, the level of extracted variance was 64.1%, with a redundancy of 34.8%. For the genotypic data, the extracted variance reached 100%, with a redundancy of 40.7%. The wet gluten content showed the highest partial correlation values with genetic populations K1–K6, followed by heading, nitrogen content, Zeleny test, and starch content. A graphical representation of the correlation analysis is displayed in Figure 4a. Two clusters represent spring and winter type of wheat accessions.

A slightly lower correlation resulted from the CCA between the genotype and phenotype data, when only rust resistance variables were used ($R = 0.58$; $p < 0.01$). For resistance to rust disease, the level of extracted variance was 100%, with a redundancy of 16.1%. For the genotypic data, the extracted variance reached 81.0% with a redundancy of 13.8%. While variables related to Pg resistance showed negative partial correlations (-0.80 and -0.50 for evaluation and for content, respectively), variables related to Pt resistance showed

positive correlations (0.28 and 0.56 for evaluation and for content, respectively). The plot (Figure 4b) shows two overlapping clusters of winter and spring wheat accessions. This can indicate that spring wheat was generally more resistant to leaf rust, whereas winter wheat was more resistant to stem rust within the studied wheat panel.

3.4. Marker-Trait Associations

LD was calculated using 13,274 SNP markers. Of the 328,564 marker pairs, 47,984 had $r^2 > 0.2$, corresponding to 14.6%.

For a deeper insight into the diversity, genome-wide association mapping (GWAS) was performed using two models—a general linear model (GLM) and a mixed linear model (MLM)—both with two types of data: the PCA and Q matrix as covariates. A Manhattan plot is shown for an example of GLM PCA model in Figure 5. In total, all four combinations of model and data matrix revealed 300 marker-trait associations (MTAs), of which 153 were associated with stem rust, 27 with leaf rust, 14 with heading time, 40 with nitrogen content, 17 with Zeleny test, five with wet gluten content, and 44 with hardness. No association was found for plant height or starch content (Table S3).

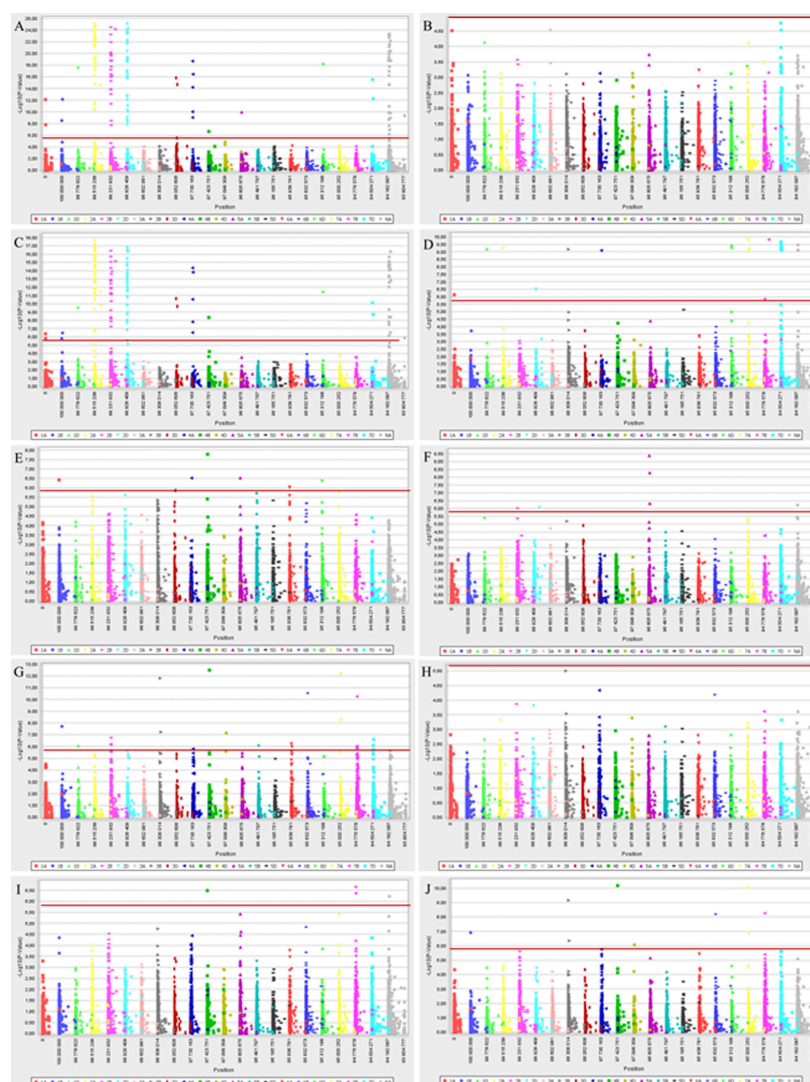


Figure 5. Manhattan plot for rust disease resistance and agronomical traits in 185 wheat accessions using the GLM PCA model. (A) Pg evaluation; (B) Pt evaluation; (C) Pg content; (D) Pt content; (E) hardness; (F) heading; (G) nitrogen compound content; (H) starch content; (I) wet gluten content; (J) Zeleny test. The various chromosomes are indicated by different colours. The horizontal red line is drawn at a specific y-value to indicate the threshold of statistical significance.

In the case of rust resistance, stem rust MTAs were obtained by combining 150 MTAs detected for Pg evaluation and 145 MTAs for Pg content, whereas leaf rust associations were identified only between genotype and Pt content (27 MTAs) but nearly all of them (23) were supported by all four combinations of model and data/covariates. Most MTAs found for rust resistance were located on chromosomes 2A, 2B, and 2D for Pg, and on chromosome 7D for Pt. BLAST analysis of significant SNP sequences revealed 128 protein-coding genes overlapping the MTAs for rust resistance identified in this study (Table S3). Several of them (47 genes) were related to plant resistance genes, namely an ABC transporter family member, an LRR receptor like protein kinase, and a disease resistance protein RGA.

4. Discussion

The long-term breeding process leads to a considerable increase in crop efficiency, but at the cost of reduced genetic variability [9,18]. This genetic homogenization creates a “fragility trap”, in which crops are increasingly susceptible to rapidly evolving biotic and abiotic stresses. On the other hand, high genetic diversity presents an opportunity to select for wide adaptations to varied constraints and traits. As rust diseases represent a constant threat of wheat yield and grain quality, the variability host plant response to the pathogens *P. triticina* and *P. graminis* was the key criterion for selection of the wheat panel in this study. Not only resistance, but also yield, grain quality, and other traits are important for breeders. Therefore, we considered these traits in our variability study, and we collected grain quality data in addition to rust disease evaluation, plant height, and heading time. All monitored traits are polygenic and multifactorial. Although the design of our experiments does not allow us to comment on the causal relationships between wheat rust resistance and grain quality parameters, it does allow us to more comprehensively assess the variability of the set of wheat genotypes. Therefore, a more holistic, multi-trait selection approach is needed [19].

ANOVA results for rust disease traits showed that the interaction between genotype and environment ($G \times E$) was significant. The impact of genotype was not dominant, but it was highly statistically significant. A p value < 0.001 confirms the presence of significant genetic differences in resistance among the tested lines.

In accordance with previous results, the Principal Component Analysis (PCA) revealed that even when genotypes were specifically selected for disease resistance, the first PCA factor, representing 40.6% of the variability, was linked to heading time and quality parameters, such as nitrogen content, wet gluten content, Zeleny test, and starch content. A certain overestimation of grain quality characteristics is due to their mutual correlation. The second and third factors already represent resistance to grass and wheat rust. Although the mutual correlation of some agronomic traits plays a role here, for breeders, this means that breeding for some traits is simpler, as a sufficient level of genetic variability is present, as was the case for grain quality traits in our wheat work collection. However, other features, such as rust resistance in our case, require breeders to seek new sources of genetic diversity for successful breeding.

The average PIC value obtained in this study when using DArTs on 185 wheat accessions was 0.24. In the context of DArT technologies, this is not a high value, but it is sufficient for a genetic diversity study. A similar value was found by Chao et al. [20] in durum wheat (0.26). Liu et al. [21] presented a higher value of PIC (0.31) for 723 Chinese wheat landraces. Given that landraces carry much more variability than modern cultivars [18], our PIC value can be considered relatively high.

Population structure was generally weak among the accessions. It reflected the type of wheat (spring or winter) and the impact of genetically distant genotypes used in breeding rather than the origin of accessions. The multidimensional scaling (MDS) analysis validated

the population structure, identifying two primary and six secondary subgroups. While the presence of two main clusters was most obvious, accounting for all six sub-clusters was essential to minimize false-positive associations.

Similarly to Liu et al. [21], a high correlation between phenotype and genotype was found, especially when all variables were considered ($r = 0.89$; $p < 0.001$). Grain quality traits revealed higher partial correlations, which is in accordance with previous results. For a deeper insight into the relationship between phenotypes and genotypes, we used GWAS.

The GWAS analysis identified a total of 300 significant MTAs associated with the 11 agronomic traits assessed. Unlike the results of PCA and CCA analyses, GWAS showed a higher number of MTAs for variables related to rust resistance (60%). It corresponds to the initial strategy of choosing a wheat panel with the widest possible range of resistance levels to leaf and stem rusts. Other traits were monitored to prevent a risk of crop genetic erosion [9].

Most MTAs related to stem rust resistances (153 MTAs) were shared by both variables, Pg evaluation and Pg content, as they represent wider range of response to fungus attack. This is also related to the fact that resistance to stem rust is controlled by numerous genes, including by 60–70 known *Sr* genes [22]. In our study, 29 putative genes significantly associated with stem rust resistance represent putative resistance proteins such as RGA, LRR domain protein kinases, or ABC transporter proteins. It is known that resistance to stem rust can be based on *NBLRR* (nucleotide-binding leucine-rich repeat) genes, which directly or indirectly recognize pathogen effectors and trigger a hypersensitivity reaction involving cell death at the site of infection [23]. Another strategy involves different mechanisms, such as sugar transport or ABC transporters, which control resistance not only to stem rust, but also to stripe rust, powdery mildew, and spot blotch [24]. Recent research published in the journal *Science* by Chen et al. [25] revealed the mechanism of the *Sr62* gene, which requires the cooperation of two neighbouring genes for full function—a tandem kinase (*Sr62TK*) and an NLR protein (*Sr62NLR*). Using modern sequencing methods, other important genes, such as *Sr43*, a protein kinase with unknown domains, and *Sr60* were cloned [26,27].

Plant resistance gene analogues (RGAs) are a large class of potential resistance genes that have conserved domains and structural features that play specific roles in pathogen resistance [28]. Well-known RGAs are nucleotide-binding site leucine-rich repeats, receptor-like kinases, and receptor-like proteins. In wheat, five RGAs have been detected so far. Two named *Sr33* and *Sr35* were identified as resistance genes against stem rust [29,30]. Three represent the genes *Lr1*, *Lr10*, and *Lr21* and they contribute to wheat resistance to leaf rust [31–33]. In our study, only 27 MTAs associated with leaf rust were identified. This is due to the lower variability of wheat accessions to Pt attack. Unlike Pg, leaf rust is less aggressive, and medium attack dominates, which is related to the fact that many QTL are involved in the plant's response [23]. Moreover, no association was found with the variable Pt evaluation; only with Pt content. Further analyses showed an understatement of visual evaluation compared to Pt content, which is related to the dynamics of the Pt attack. Thus, Pt content assessed by real-time PCR may represent leaf rust infection more accurately. The influence of the fungus attack dynamics to disease evaluation and GWAS will be the subject of further study.

A non-specific resistance mechanism may play an important role in rust disease. Five MTAs were identified as berberine bridge enzyme-like genes. Berberine bridge enzyme is a key enzyme in the biosynthesis of isoquinoline alkaloids. Research has shown that these berberine bridge-like enzymes also occur in wheat. They may play a role in defence against fungal pathogens, including rusts, by participating in the oxidation of precursors in the cell wall, thereby strengthening it by lignification and preventing penetration by pathogen hyphae [34]. Further, 11 MTAs were identified as acetyltransferase *At3g50280-like*

genes. In wheat, these acetyltransferases are mainly studied in connection with induced resistance. They are likely involved in rapid adaptation to pathogen attack through modification of hormonal pathways [35]. Therefore, the discovery of similar markers through GWAS analysis offers a promising pathway for breeding. Because this was our initial study of GWAS, further analyses will focus on MTAs' functional validation and validation in an independent wheat population.

5. Conclusions

In conclusion, the variability of a panel of 185 wheat accessions was evaluated using DArT markers, leaf and stem rust resistance and several agronomical trait evaluations. The results showed that both types of traits affect the variability of wheat accessions. On the genome level, six populations ($K = 6$) were identified, four of them are predominant in winter wheat and two in spring wheat with a relatively high level of admixture between populations. A high correlation was found between genotype and phenotype features ($R = 0.89$). A deeper analysis of variability using GWAS identified 300 associations between traits and DArT markers. Although none of the MTAs correspond to previously described genes for resistance to wheat to stem and wheat rusts, some of them (47) are associated with non-specific resistance. For breeders, this means that a selected set of wheat genotypes allows breeding for resistance to stem and wheat rusts without reducing the level of grain quality traits. Non-specific resistance genes can play a key role in this selection. However, additional trials are needed to validate the identified MTAs and possibly convert them to MAS.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/agronomy16141322/s1>, Table S1: List of wheat accessions including phenotype data; Table S2: Genotype data; Table S3: List of MTAs with blast results; Table S4: Wheat accessions cluster membership computed by Structure.

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Abbreviations

The following abbreviations are used in this manuscript:

DArT	Diversity Arrays Technology
PCR	Polymerase Chain Reaction
Pg	<i>Puccinia graminis</i> , stem rust
Pt	<i>Puccinia triticina</i> , leaf rust
QTL	Quantitative Trait Locus

GWAS	Genome-Wide Association Study
PCA	Principal Component Analysis
ANOVA	Analysis of Variance
CCA	Canonical Correlation Analysis
MDS	Multidimensional Scaling
PIC	Polymorphism Information Content
LD	Linkage Disequilibrium
GLM	Generalized Linear Model
MLM	Mixed Linear Model
MTA	Marker-Trait Association

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