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Genetic Diversity of Clinal Freezing Tolerance Variation in Winter Wheat Landraces

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Abstract: Wheat (*Triticum aestivum* L.) is a major cereal crop grown across a wide range of environments, but its productivity around the world is challenged by various biotic and abiotic factors. Wheat landraces from around the world are a source of unexploited genetic diversity that can be essential for modern wheat-breeding programs in search of resistance to abiotic stresses like freezing tolerance. This genetic diversity study of 553 winter wheat landraces based on single-nucleotide polymorphisms (SNPs) revealed separate clusters of landraces related to the latitude of origin. Linkage block analysis revealed genomic regions with specific alleles skewed towards landraces from higher latitudes, suggesting that migration to higher latitudes resulted in the fixing of specific alleles. Electrolyte leakage was used to measure the tolerance of freezing to −14 °C, −16 °C, and −18 °C of 192 landraces. There was a significant negative correlation between latitude and electrolyte leakage, with an R^2 value of 0.14, ($p < 0.0001$), in a regression analysis indicating greater freezing tolerance in landraces from higher latitudes. Genome-wide association studies identified regions in chromosomes 4A and 6A associated with higher latitudes and freezing tolerance, respectively. Landraces with freezing tolerance may be useful in developing new germplasm as novel sources of greater cold hardiness.

Keywords: *Triticum aestivum*; landraces; freezing tolerance; electrolyte leakage; genome-wide association studies

1. Introduction

Wheat, *Triticum* spp., is a major cereal crop that is grown across a wide range of environments covering more land than any other commercial crop in the world, mainly concentrated within the latitudes of 30 and 60° N and 27 and 40° S [1,2]. Cultivation of wheat started around 10,000 years ago in the Fertile Crescent during the Neolithic Revolution when the hunter–gatherer lifestyle transitioned into agriculture-based societies. Agriculture expanded further from the Fertile Crescent, and cultivated crops and domesticated animals spread together [3]. Movement to new climates accompanied by natural polyploidization led *Triticum* spp. to increase in genetic diversity and adapt to a wide range of environmental regimes [4]. The Karacadag region of southeast Turkey in the Fertile Crescent is regarded as the "core area" where several wild progenitors of various domesticated cereals and legumes appeared together. Archaeological evidence indicates that the core area was an active cultural center from which the culture along with crop domestication spread to other parts [3,5,6]. The first cultivated wheats were einkorn wheat and emmer wheat [7]. Natural stands of wild diploid einkorn

(*T. boeoticum*) and wild tetraploid emmer (*T. dicoccoides*) are still found widely across the Fertile Crescent [8,9]. Phylogenetic analysis based on amplified fragment length polymorphism (AFLP) fingerprinting indicated that cultivated einkorn (*T. monococcum*) is closely related to wild einkorn wheat from the Karacadag Mountains, suggesting that the Karacadag region may have been the site for first domestication of einkorn wheat [8,10].

Domestication of tetraploid emmer wheat, which likely took place in the southeastern part of present-day Turkey, was an important event in the process of wheat evolution [9]. The emmer wheat was derived by natural hybridization of wild diploids, with the A genome contributed by *Triticum urartu* (2*n* = 14, AA) [11,12], and the B genome by a relative of *Ae. speltoides* (2*n* = 14, BB) [13]. Wild emmer has brittle ears that shatter at maturity and is different from the domesticated emmer wheat (*T. dicoccum*), which has a non-brittle rachis with hulled seeds [3]. During the Neolithic and early Bronze ages, emmer wheat was the principal crop in the Fertile Crescent [3,14]. Mutation events in hulled emmer led to the development of free-threshing tetraploid wheat *T. turgidum* L. [15,16], which is now cultivated widely around the world.

Common or bread wheat is an allohexaploid derived from the natural hybridization of the tetraploid wheat with the diploid goatgrass, *Aegilops tauschii* (2*n* = 14, DD) [17–19]. The evolution of hexaploid wheat began outside the Fertile Crescent around 9000 years ago, when tetraploids reached the range of *Aegilops tauschii* (DD) extending from Armenia to the coastal areas of the Caspian Sea [10,14,19]. Several events of natural hybridization may have contributed to the evolution of modern-day hexaploid wheat [20]. The first hexaploid wheat was similar to *T. spelta* (hexaploid, hulled wheat), which may have undergone hybridization with free-threshing tetraploids to give rise to free-threshing hexaploid forms [17]. The *Q* gene, located at the long arm of chromosome 5A, is responsible for the free-threshing character and is also pleotropic to other important traits of domestication including rachis fragility, shape and softening of glumes, spike length, and culm height [3,21,22]. The *Q* gene was an important factor in wheat evolution, leading to the rapid spread of free-threshing wheat worldwide [22].

Agriculture spread east–west rather than north–south as movement to the north or south requires clinal adaptation to different day lengths and climates that occur with the change in latitude [10,23]. The spread across central Europe, from Hungary to Belgium and eastern France, was particularly rapid [24]. While migrating to new uncolonized areas, people carried crops and farming knowledge with them. Establishment of techniques to adapt crops and livestock may have helped in the rapid spread of agriculture into new areas with cultivable lands [25]. In addition to suitable environment, cultural selection of specific crops among the communities may have played a role in the transfer of fewer sets of crops to the new cultivable areas from the earlier colonized areas [24]. Introduction of the crop plant to new environments and territories allows for the selection of new mutations that establish geographic races that are expected to display phenotypic variation, particularly across latitudes [26,27]. Solar radiation and temperatures decrease with the increase in latitude. With the movement of crop domestication towards the north, the crops were selected for cold hardiness in order to adapt to lower average temperatures and harsh winters at those latitudes [28,29].

Exotic germplasm, including wild species and wheat landraces from different parts of the world, are a potential source of unexploited genetic diversity that can be introduced into modern wheat breeding [30,31]. Genetic information on these germplasms can provide direct evidence for the adaptation of crops to climatic conditions and can shed light on the genomic basis that influenced the agricultural spread into different regions [23,32].

Wheat is grown at latitudes as high as 60° N, but many wheat cultivars suffer significant yield losses due to freezing injury and a lack of winter hardiness [33–35]. Genetic control of cold tolerance in wheat is a complex trait and quantitative trait loci (QTL) or chromosome regions associated with cold tolerance have been identified on chromosomes 1A, 1D, 2A, 2B, 3A, 5A, 5B, 6A, 6B, 6D, and 7B [36–40]. Perhaps the most significant chromosomal region impacting freezing tolerance is the Fr-A2 locus, a region of chromosome 5A that has been investigated many times [41–46]. This region contains many

C-repeat Binding Factor (CBF) genes encoding transcription factors known to be instrumental in the regulation of many cold-responsive genes and may account for nearly 25% of the variation in freezing tolerance in some populations [46]. Other genes contributing to cold tolerance and overwintering ability are the genes controlling vernalization requirement (*VRN*) and photoperiod sensitivity (*PPD*). Vernalization requirement and the ability to acquire freezing tolerance are intricately associated [47], as are photoperiod sensitivity and the ability to express genes enabling low-temperature tolerance [48]. *VRN* genes are mapped to the long arms of homeologous group 5 chromosomes 5A, 5B, and 5D [49]. *PPD-1* genes are located on the homeologous chromosomes group 2 [49] and are named *PPD-A1*, *PPD-B1*, and *PPD-D1* [50] on 2A, 2B, and 2D, respectively.

Although many genomic regions associated with freezing tolerance have been identified and are being exploited in breeding programs, sufficient winter hardiness of available cultivars continues to be problematic [35]. Greater diversity of sources of cold-tolerant genotypes may contribute to further development of freezing tolerance and winter hardiness; wheat landraces from around the world are potential sources of this diversity. Therefore, the objective of this research were to assess genetic diversity of winter wheat landraces and to determine the genomic regions associated with the necessary adaptation to colder climates that occurred in wheat with migration from the Fertile Crescent to northerly latitudes.

2. Materials and Methods

SNP marker analysis of winter wheat landraces. The USDA-ARS National Small Grains Collection (NSGC), Aberdeen, ID, provided 553 winter wheat landraces (Table S1) for this study, collected from 31 countries with collection sites ranging from latitudes 27.5° N to 60° N and longitudes 9° W to 129.6° E. These landraces were included in a single-nucleotide polymorphic (SNP) genotyping experiment using the Illumina 9K wheat iSelect assay (http://www.illumina.com) conducted for the Triticeae Coordinated Agricultural Project (http://www.triticeaecap.org) by the USDA-ARS, Biosciences Research Lab, Fargo, North Dakota. The SNPs were based on expressed sequence tags (ESTs), and therefore originated in the transcribed regions of expressed genes. Data for 9000 SNP markers was obtained, but after filtering monomorphic and low-quality SNPs, and markers with minor allele frequencies (MAF) of less than 5%, data for 5476 informative SNP markers were retained and used for further analysis in this study.

Landrace accessions were divided into 10 groups based on latitude of origin. Principal coordinates analysis (PCoA) was performed using the covariance-standardized method based on distance matrix of the landraces in GenAlex 6.41 [51]. Population structure of 553 landraces was inferred using STRUCTURE software [52] version 2.3.3. For STRUCTURE analysis, in addition to 5% cutoff for missing data and MAF, only one marker from same position, based on 9K SNP consensus map [53], was included. Altogether 591 SNPs at an interval of 4 cM (centimorgans) were selected covering the whole wheat genome. An admixture model was used with correlated allele frequencies. The burn-in period was set at 50,000 and MCMC (Markov chain Monte Carlo) repetitions at 100,000. Analysis was replicated five times for each number of assumed clusters (K) from K = 1 to 10. The output data from STRUCTURE were assessed through STRUCTURE HARVESTER [54], where the best number of clusters (K) was determined by the Evanno method [55]. The data for the best K were run on program CLUMPP (CLUster Matching and Permutation Program [56]) to correctly align the clusters labeled from all five replications in STRUCTURE.

The landrace accessions were divided into two groups based on the latitude of origin (below and above 40° N latitude) and linkage block analysis was performed across the whole genome based on the consensus map of 9K SNPs [53]. Two groups were separated considering remarkable genetic variation between landraces below and above 40° N latitude conferred by PCoA analysis. Genotypic data (SNPs called A or B) for all the accessions were normalized based on a randomly selected accession (PI 351046). All of the SNP calls for PI 351046 were changed to "A" and calls for other genotypes were

changed accordingly. Normalization helped with better visualization of the allelic variation in linkage blocks of the two groups.

SNP tags of interest were further investigated by identifying their physical location using the search functions in the Triticeae toolbox (https://triticeaetoolbox.org), then examining the corresponding annotation provided in the wheat genome assembly at Ensembl (http://archive.plants.ensembl.org/Triticum_aestivum/Info/Index). When necessary, further searching of the GenBank database (http://www.ncbi.nlm.nih.gov) was carried out using BLASTn tool.

Electrolyte leakage test for freezing tolerance. The electrolyte leakage method measures damage to the cell membrane by detecting leaked cell contents when cells are exposed to stress conditions such as freezing temperature [57]. Increased permeability of injured cells with ruptured cell membranes release cell contents at a greater rate in comparison to a healthy intact cell in water [57]. Thus, lower electrolyte leakage recorded after freezing treatment indicated that the tissue was more cold-tolerant. The electrolyte leakage method has been used since the 1930s for many experimental purposes, such as to estimate the hardiness of plants [58] and compare root and shoot injuries of woody plants due to frost [59]. Bajji et al. (2002) [60] used the electrolyte leakage technique with durum wheat as a predictive test to evaluate water stress tolerance, and Cuevas et al. (2015) [61] used electrolyte leakage to evaluate freezing tolerance of young wheat plants. From the 553 winter wheat landraces described above, 192 from 29 countries within the latitudes ranging from 27.5° N to 60° N were selected for electrolyte leakage analysis.

Plants were grown in Sunshine Mix LC1 planting medium (Sun Gro Horticulture, Bellevue, WA, USA) in six-container packs (Model 1020, Blackmore Co., Belleville, MI, USA). Each of the six containers in each pack was planted with 20 seeds of one of the wheat lines to be tested. Seeds were germinated and plants were grown at 22 °C in a growth chamber (Model E15, Conviron, Pembina, ND, USA) under cool, white fluorescent lights (about 300 μmol m^{-2} s^{-1} at the soil surface) with a 16-h photoperiod until the seedlings reached the three-leaf stage. The plants were then transferred to 4 °C with a 16 h photoperiod (about 250 μmol m^{-2} s^{-1} at mid-plant height) for five weeks to induce cold acclimation prior to freezing/electrolyte leakage tests.

In preparation for the tests, the plants and planting medium from one cell were removed from the cell packs and immediately submerged in ice water. The soil was rinsed from the plants to expose the crowns of the plants, the plants were clipped just below the crowns, removing the roots, residual caryopses were removed, and the shoots of the plants were removed at the top of the lowermost leaf sheath, resulting in about 5 cm of crown and "stem" region of the plants remaining. These plants were briefly placed in ice water to rinse away cellular fluid from the cut surfaces; they were blotted dry and individual plants were placed in 15 mL polypropylene, screw-cap tubes and immediately placed on ice. Each of the 192 wheat lines tested was represented by three plants in each test.

The tubes containing the plants were placed in racks that were transferred from the ice to a Microclimate® benchtop environmental chamber (Cincinnatti SubZero, Cincinnatti, OH, USA) at 0 °C. The temperature was lowered to the target temperature (−14, −16, or −18 °C) at a rate of 2 °C h^{-1}. The temperature in the chamber was monitored using thermistor probes and a Hydra Data Bucket temperature monitor (Fluke Inc, Everett, WA, USA). When the temperature had been maintained at the target temperature for 1 h, the tubes containing the plants were removed and immediately plunged into crushed ice. The tubes were held on ice for 30–60 min. to allow the temperature of the plants to slowly return to 0 °C, then 5 mL of ultrapure water were added to each tube and tubes were incubated at room temperature with shaking at 80 rpm for 12 h. The conductivity of the solution was then measured using a benchtop conductivity meter (Hanna Instruments, Woonsocket, RI, USA, Model HI216). The tubes were then immersed in liquid nitrogen until frozen solid and then allowed to thaw and return to room temperature. The conductivity of the solution was again measured and cell damage was estimated as the ratio of the conductivity of the solution before freezing in liquid nitrogen, relative to the conductivity of the solution after freezing in liquid nitrogen (complete cellular disruption).

The entire experiment was repeated and the electrolyte leakage data were analyzed with PROC GLM and PROC REG available in SAS (Statistical Analysis System, Cary, NC, USA). The damage estimates (proportion of total cellular leakage) were arcsin-square root transformed for analysis purposes, but are shown in the original scale in this report. Least-square means were compared using the pdiff option of PROC GLM. Geographic distribution of relative freezing tolerance was visualized using spline regression and the background world map option available in JMP software (http://www.jmp.com).

Genome-wide association analysis. Genome-wide association studies (GWAS) was performed by using FarmCPU package [62]. GWAS were conducted in two phases. The first phase was to identify the genetic loci associated with population structure (based on principal component PC1, calculated in R software) and latitude in 553 landraces. The second phase used two genetic loci associated with population structure and one locus associated with latitude as covariates, and freezing tolerance (measured as electrolyte leakage) as observations in 192 landraces. For each landrace, the electrolyte leakage scores for all replications at all three temperatures were averaged and analyzed for GWAS. The threshold *p*-value for significant marker-trait association was based on a Bonferroni-corrected threshold with 0.01.

3. Results

3.1. Genetic Diversity of Winter Wheat Landraces Based on SNPs

In the PCoA of 553 winter wheat landraces based on 5476 SNPs, the first and second axes of the PCoA accounted for 58.67% and 12.83% of the total genetic variation of landraces, respectively (Figure 1). When landraces were labeled according to the region of origin the landraces from Western Asia formed a dense group at the lower right quadrant and spread across upper right and left quadrants (Figure 1). East and South Asian landraces clustered within the West Asian spread. All the European landraces were in a distinct cluster at the lower left quadrant except for a few East and South European landraces. Interestingly, when the landraces were labeled based on ranges of latitudes of their origin, it became apparent that there was a separate cluster containing nearly all of the landraces from latitudes higher than 40° N at the lower left quadrant (circled on Figure 1) distinct from all the other landraces from latitudes lower than 40° N. Population structure analysis on 553 winter wheat landraces revealed the best $K = 2$ based on the delta-K method. The majority of the landraces from lower latitudes (<40° N) grouped together in one cluster and landraces from higher latitudes (>40° N) grouped into another (Figure S1).

Histograms for allele frequencies for all of the SNPs considering two groups of winter wheat landraces (from below or above 40° N latitude) revealed linkage blocks with allele frequencies highly skewed towards one of the groups, suggesting them to be from genomic regions that differentiated with movement towards the north. These genomic regions included linkage blocks from chromosomes 2A, 4A, 5B, and 6A. In chromosome 2A, the majority of SNPs between 116 cM to 125 cM had ≥80% of 'A' allele in landraces from higher latitudes and ≤20% of 'A' allele in landraces from lower latitudes. Similarly, 53 cM to 62 cM in chromosome 4A, 58 cM to 60 cM in chromosome 5B, and at 89 cM in 6A, and 103 cM to 117 cM in chromosome 6A had ≥80% of 'A' allele in landraces from higher latitudes and ≤20% of 'A' allele in landraces from lower latitudes. Examples are shown in Figure 2.

Genome-wide association studies based on 5476 SNPs identified SNPs associated with population structure and latitudes. Several SNP markers from all chromosomes showed association with population structure, most strongly, SNP IWA3431 from chromosome 2A and IWA6597 from 4A. Further information on these SNP tags including possible gene identification of the corresponding transcripts is provided in Table 1.

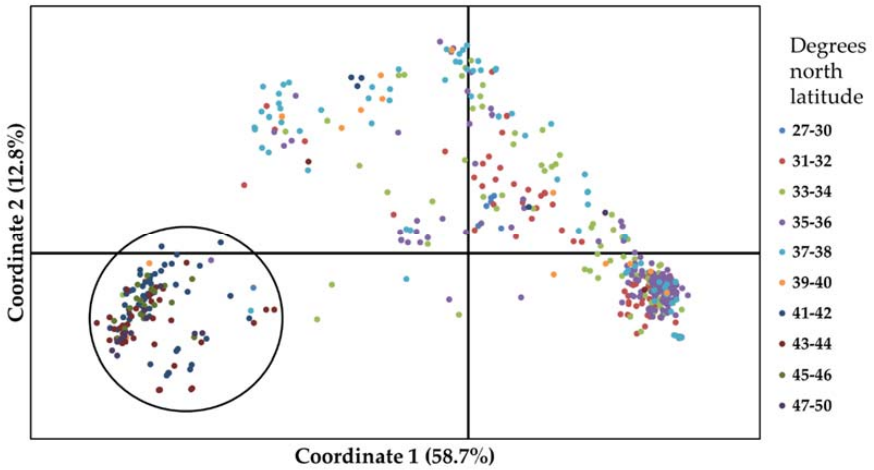

Figure 1. Principal coordinates analysis (PCoA) based on 5476 SNP markers on 553 winter wheat landraces. Landraces are color coded based on ranges of latitudes of their origin. The circled accessions indicate clustering of most of the accessions from ≥40° north latitude.

Table 1. Genome-wide association studies results showing significant SNPs for population structure, latitude, and electrolyte leakage (as a measure of freezing tolerance) in winter wheat landraces.

SNP [a]	CHR [b]	cM [c]	MAF [d]	Obs [e]	*p*-Value	Trait	Possible Gene Identification [f]
IWA3431	2A	117.57	0.37	553	3.02×10^{-124}	Population structure	Translation initiation factor
IWA6597	4A	57.56	0.26	553	1.11×10^{-107}	Population structure	Unknown function, transmembrane domains
IWA7271	4A	46.27	0.23	553	1.02×10^{-50}	Latitude	phosphatidylinositol *N*-acetyglucosaminyltransferase
IWA1050	6A	85.53	0.41	192	1.52×10^{-8}	Electrolyte leakage	chlorophyll a/b binding protein

[a] single-nucleotide polymorphic marker; [b] chromosome number; [c] position of the SNP marker in the chromosome in centimorgan (cM); [d] minor allele frequency; [e] number of observations; [f] possible gene identification based on annotations in the wheat genome assembly at Ensembl (http://archive.plants.ensembl.org/Triticum_aestivum/Info/Index) or a BLAST search of the GenBank database (http://www.ncbi.nlm.nih.gov).

SNP tag IWA7271 from chromosome 4A was strongly associated with latitude ($p < 10^{-50}$, Table 1). The wheat genome assembly at Ensembl revealed that SNP IWA7271 is from within an exon of a gene designated as Traes_4AS_1658FFBB2.1, consisting of five exons and three introns. The five exons were manually assembled into a continuous sequence and used to query the GenBank database using the nucleotide BLAST function. The result indicated the DNA transcript sequence containing SNP tag IWA7271 is 70% identical over 2957 base pairs (Expect value = 0.0) to a gene identified from *Zea mays* encoding a subunit of phosphatidylinositol *N*-acetyglucosaminyltransferase (Sequence ID: XM_008660105.2). The translated protein product of Traes_4AS_1658FFBB2.1 also matched the *Zea mays* amino acid sequence of the phosphatidylinositol *N*-acetyglucosaminlytransferase gene, accession AQL07576, with an Expect value of 0.0 (using protein BLAST). These observations suggest that SNP tag IWA7271 occurred within a gene that may be involved in cell membrane function and glycolipid biosynthesis.

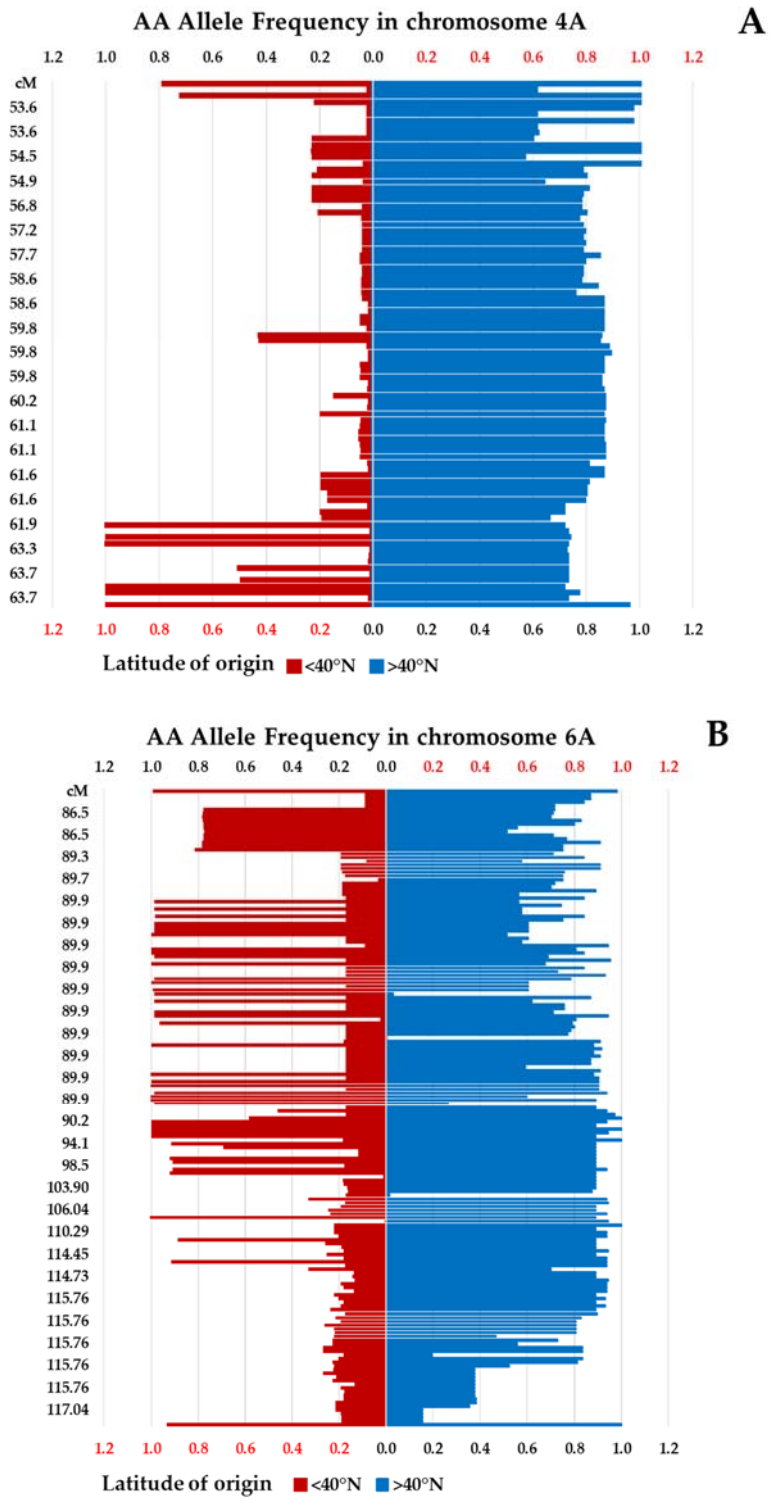

Figure 2. Linkage block analysis for the distribution of allele frequencies of 553 winter wheat landraces from higher (>40° N) and lower (<40° N) latitudes in (**A**) chromosome 4A and (**B**) chromosome 6A.

3.2. *Electrolyte Leakage Test*

The mean electrolyte leakage, expressed as the conductivity following the freezing test as a proportion of conductivity after freezing in liquid nitrogen, was 0.63 at −14 °C, 0.74 at −16 °C, and 0.78 at −18 °C. The analysis of variance indicated that 53% of the variation in the electrolyte leakage measurements can by ascribed to the latitude from which the wheat landraces were collected

(Table 2). The freezing challenge temperature the plants were exposed to accounted for 41% of the variation; replications and subsamples accounted for 6% (Table 2). Even at the lowest treatment temperature of −18 °C, there were landraces with electrolyte leakage measures of 0.3 to 0.5, suggesting those landraces may be highly cold-tolerant (Figure S2). The regression analysis based on all of the data indicated a highly significant negative relationship of latitude and electrolyte leakage with an R^2 value of 0.14, ($p < 0.0001$), indicating a general trend toward less damage (greater cold tolerance) in landraces from higher latitudes (Figure 3). A contour plot of electrolyte leakage superimposed on the world map clearly showed a relationship of greater freezing tolerance with increased latitude of origin (Figure 4).

Table 2. Analysis of variance reporting significant effect of the geographic latitude of origin and the test temperature on freezing tolerance of 192 winter wheat (*Triticum aestivum* L.) landrace accessions.

Source of Variation	df [a]	Sum of Squares [b]	% Explained Variation
Latitude	1	21.6 ***	52.5
Test temperature	2	17.0 ***	41.5
Replications	1	2.3	5.6
Subsamples	2	0.2	0.4
Model	6	41.1	
Error	2949	258.4	

[a] degrees of freedom; and [b] stars (***) indicate significance at $p < 0.001$.

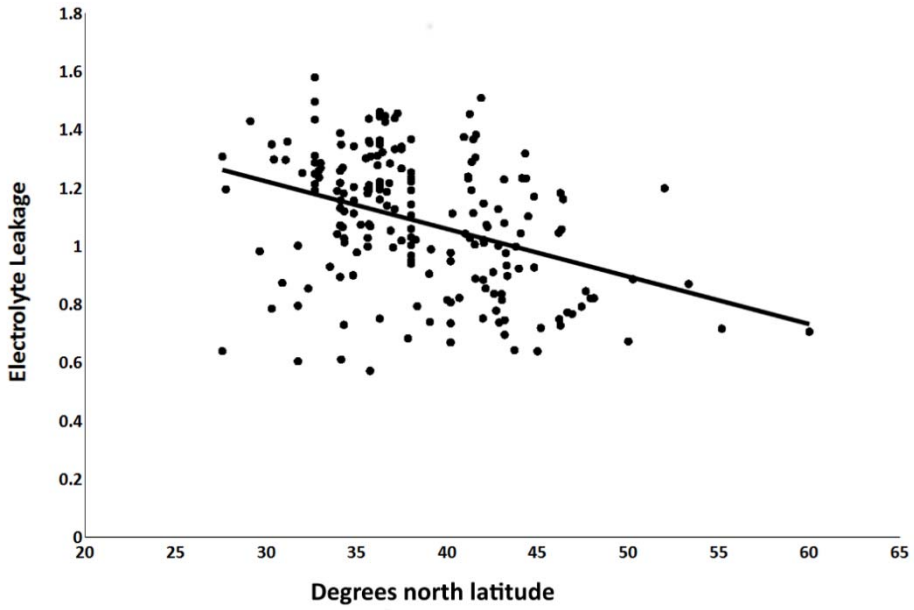

Figure 3. Regression analysis of freezing tolerance on latitude of origin of 192 winter wheat landraces. Freezing tolerance was measured as electrolyte leakage of cold-acclimated seedlings following freezing to −14, −16, or −18 °C. Electrolyte leakage was expressed as the arcsine-transformed proportions of leakage after the freezing challenge relative to leakage after freezing in liquid nitrogen. $R^2 = 0.14$, $p < 0.0001$.

A genome-wide association study based on the 5476 SNPs identified SNPs associated with electrolyte leakage, with SNP IWA1050 from chromosome 6A the most significant (Figure 5). Among the A or G allele of IWA1050, allele A was favorable for freezing tolerance. The wheat genome assembly at Ensembl revealed that SNP IWA1050 is from within a gene designated as Traes_6AS_58D403D66, which encodes a chlorophyll a/b binding protein.

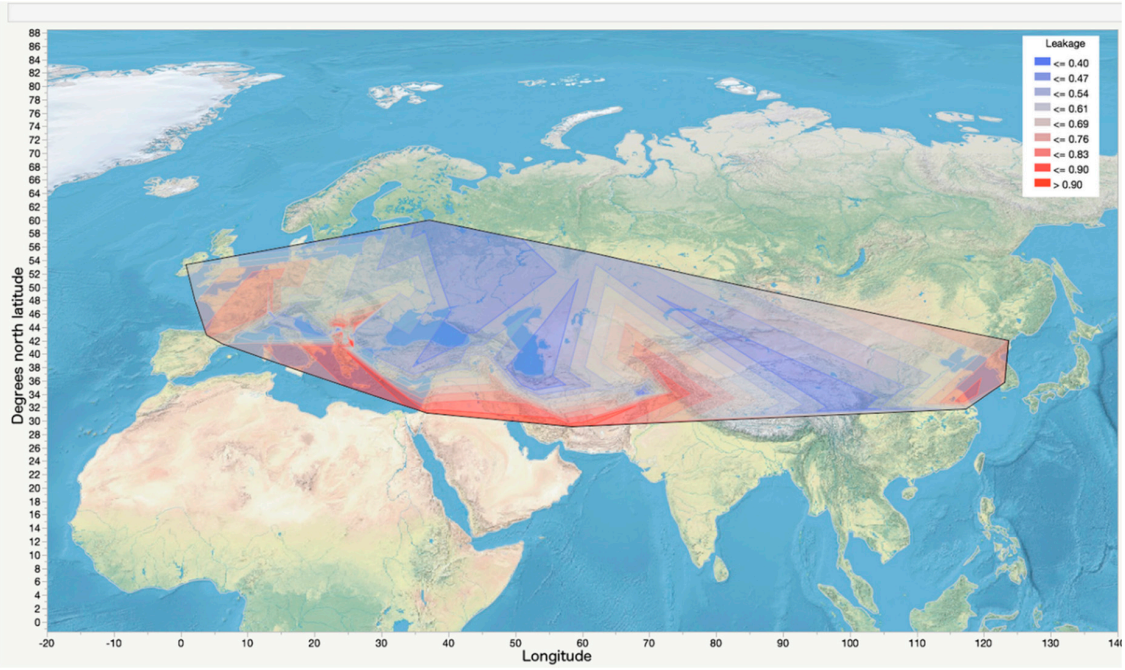

Figure 4. Contour plot of spline regression of freezing tolerance on latitude of origin of 192 wheat landraces collected from latitudes 27.5° N to 60° N and longitudes 9° W to 129.6° E, superimposed on a map of the world. Freezing tolerance was defined as electrolyte leakage following freezing to −14, −16, or −18° C, expressed as a proportion of leakage following immersion in liquid nitrogen; lower numbers indicate greater freezing tolerance.

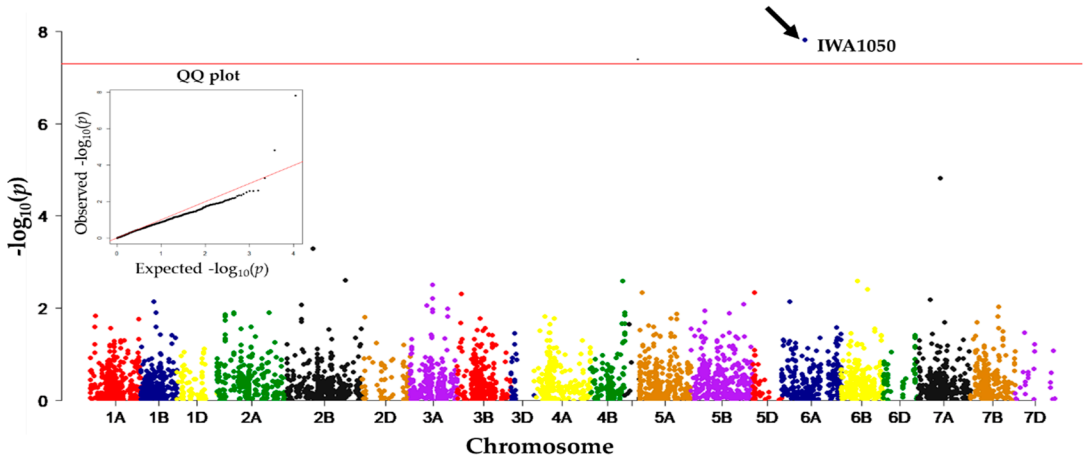

Figure 5. Manhattan plot, and QQ (quantile-quantile) plot, from genome-wide association studies for freezing tolerance (measured as electrolyte leakage) in 192 winter wheat landraces using 5476 SNPs. Deflation of the QQ plot was due to the use of genetic loci associated with population structure and latitude as covariates.

4. Discussion

In the genetic diversity studies of winter wheat landraces, landraces from latitudes higher than 40° N formed a separate cluster in the PCoA analysis based on 5476 SNPs, suggesting genetic variation due to adaptation to different environments. The latitudinal gradient is associated with distinct differences in climatic factors that can result in strong natural selection for local adaptation of plants. Plant species adapted to broad geographic regions have exhibited substantial variation

in morphology, physiology, and development, most obvious along the latitudinal or altitudinal gradient [63]. During the Neolithic Revolution, with the spread of agricultural practices, crop plant species spread to new geographical regions. Migration to new regions offered an opportunity for adaptation to the new environment, which in turn contributed to the genetic variation in the adapted species. We observed this type of genetic variation among the 553 winter wheat landraces examined.

Linkage block analysis of the entire wheat genome identified regions from chromosomes 2A, 4A, 5B, and 6A to have specific alleles skewed towards landraces from higher latitudes (e.g., Figure 2), suggesting that loci on those chromosomes may have played a role in adaptation to more northerly latitudes. Similarly, GWAS identified SNPs on chromosomes 4A (IWA6597), and 2A (IWA3431) to be significantly associated with population structure, and a SNP on 4A (IWA7271) to be associated with variation in latitude of origin (Table 1). These results suggest that there were major genetic modifications in certain genomic regions of the hexaploid wheat genome when wheat was migrated to higher latitudes. Observations of linkage blocks and significant marker-latitude association in GWAS indicated that those modifications were genetically fixed on certain chromosomes and were carried along during the course of migration. As adaptation to higher latitudes also basically adapts plants to colder environments, it is possible that the linkage blocks and GWAS outputs are associated with cold tolerance. GWAS also identified a genetic region on chromosome 6A (IWA1050) to be significantly associated with freezing tolerance. The QQ plot demonstrated that the associations between freezing tolerance and the markers were deflated as genetic loci associated with population structure and latitudes were fitted as covariates.

The strong association of specific SNP tags (Table 1) with latitude of origin, population structure, or freezing tolerance may be indicative of the expression of the corresponding genes being necessary for adaptation. The association of SNP tag IWA7271, apparently from a gene encoding phosphatidylinositol *N*-acetyglucosaminyltransferase, with latitude of origin suggests that more highly-specialized membrane/lipid structures may have developed in landraces from more northerly latitudes, presumably contributing to their ability to tolerate the harsher environments. The finding of the association of freezing tolerance with an allele of SNP tag IWA1050, from a gene encoding a chlorophyll a/b binding protein, is consistent with previous findings of involvement of chlorophyll a/b binding proteins in the response of wheat plants to freezing stress [64,65]. While chlorophyll a/b binding proteins were initially discovered as part of the light-harvesting complex, recent studies have implicated these proteins in the expression of numerous traits in cereal grains, including stress responses [66–69]. Expression of these kinds of genes may result in greater intrinsic physical strength of cell walls and membranes in the landraces from higher latitudes, but also may result in differences in the ability to effectively acclimate to cold temperatures. It previously was shown that wheat lines differ in the response to the onset of low temperatures, such that some lines more rapidly and effectively acclimate to the cold than others [35].

Genetic analyses including PCoA, population structure, and linkage block analysis of these 553 winter wheat landraces from different geographical regions indicated that extensive genetic variation accompanied clinal adaptation to higher latitudes. Specific genomic regions (e.g., Figure 2) and specific genes (Table 1) appear to provide adaptive advantages. Further elucidation of the functions and efficacy of these genes and genomic regions may prove useful in expanding the geographic range of productive wheat crops.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4395/8/6/95/s1, Figure S1: Population structure of 553 winter wheat landraces, Figure S2: Freezing tolerance of 192 winter wheat landraces across three treatment temperatures and latitude of origin of the landraces, Table S1: List of 553 winter wheat landrace accessions, their country and latitude of origin, and average electrolyte leakage of 192 accessions tested for freezing tolerance.

Author Contributions: J.S.K., D.Z.S. and D.R.S. conceived and designed the experiments; J.S.K. performed the experiments; J.S.K., M.H., Z.Z., D.Z.S. and D.R.S. analyzed the data; J.S.K., D.Z.S. and D.R.S. wrote the paper.

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