Molecular Diversity within a Mediterranean and European Panel of Tetraploid Wheat (T. turgidum subsp.) Landraces and Modern Germplasm Inferred Using a High-Density SNP Array

Article

Molecular diversity within a Mediterranean and European panel of Tetraploid wheat (*T. turgidum* subsp.) landraces and modern germplasm inferred with High density SNPs array

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Abstract : High-density single nucleotide polymorphisms (SNPs) molecular markers are widely used in order to assess plant varieties and cultivars genetic variability, which is nowadays recognized as an important source of well adapted alleles for environment stresses. In our study, the genetic diversity and population genetic structure of a collection of 264 accessions of eight tetraploid *Triticum turgidum* L. subspecies were investigated using 35,143 SNPs screened with a 35K Axiom® array. Neighbor joining algorithm, discriminant analysis of principal components (DAPC) and Bayesian model-based clustering algorithm implemented in STRUCTURE software revealed clusters in accordance to the taxonomic classification, reflecting the evolutionary history and the phylogenetic relationships among *Triticum turgidum* L. subspecies. This research provides a great contribution for future phenotyping and crossing activities, improving the efficiency of recombination and allowing gene selection programs to develop durum wheat composite cross populations adapted to Mediterranean conditions.

Keywords: Axiom 35k Wheat Breeders array; Genetic diversity; Population structure; Wheat genotyping

1. Introduction

Wheat represents the third most important cereal grain and the most widely grown crop in the world [1]. Common wheat (*Triticum aestivum* L.) and durum wheat (*T. turgidum* L. ssp. *durum*) are the two subspecies predominantly cultivated, used for bread-making or leavened products (cookies, cakes and pizza) and for semolina products and pasta, respectively. In addition, both wheat species' by-product are used for animal feed production.

While bread (*T .aestivum*) wheat is hexaploid (2n=6x=42 chromosomes, AABBDD genomes), durum wheat belongs to *T. turgidum* tetraploid subspecies group (2n=4x=28 chromosomes, AABB genomes) which includes other six subspecies (*T. carthlicum*, *T. dicoccum*, *T. dicoccoides*, *T. paleocolchicum*, *T. polonicum*, *T. turgidum*) rarely grown commercially [2,3]. Many studies based on cytological and molecular analysis ascribe two different evolutionary steps to tetraploid wheat origin, which started around 10000 years ago in the Fertile Crescent [4,5]. The first divergent evolution, of which the original progenitor is unknown, gave rise to diploid species including *T. urartu* (A genome), *Ae. tauschii* (D genome), *Hordeum vulgare* (barley) and *Secale cereale* (rye) [6]. The second evolutionary process was a natural hybridization between *T. urartu* (the A genome donor) and an unknown *Triticum* species, often identified as *Aegilops speltoides* (the B genome donor), which created the wild emmer *T. dicoccoides* (2n=4X=28, BBAA genomes), the progenitor of durum wheat [7]. The history of durum evolution is the result of domestication starting from wild emmer genotypes and of a transition process from a naked emmer type to durum type [8]. Its domestication was followed by a rapid spread of durum genotypes

from the East to the West of the Mediterranean Basin [9], reaching the Iberian Peninsula around 7,000 years Befor Present (BP) [10]. Natural and human selection through thousands of years led to the establishment of wheat landraces characterized by a strong adaption to the environmental conditions and cultivation practices of different geographic areas [11]. Local traditional farming communities contributed to the maintenance of these landraces that were characterized by different qualitative and quantitative traits until the first decades of the twentieth century [12].

At the beginning of the 20th century, breeders imposed a strong selection based on commercial purposes: local landraces cultivation were progressively abandoned and replaced with improved, widely adapted and more productive semi-dwarf varieties, resulting in a reduced level of genetic diversity, especially compared to the wild ancestors [13-15]. Today, this lack of diversity is widely recognized as a limiting factor in the breeding of high yielding and stress-resistant varieties [16]. Moreover, under the current climate changes events (irregular rainfall, high temperature during the growing season, rainy storms, and drought) that negatively affect wheat cultivation, the development of new resilient varieties or composite cross populations (CCPs) adapted to different cultivation environments and low input agriculture became necessary [17-19]. Novel genetic diversity selected by breeders may be introduced in modern genotypes by the introgression of useful alleles from landraces, ancestors, or wild relatives through specific breeding programs [20-22]. Durum wheat landraces and other *Turgidum* subspecies, usually exhibit reduced productive performance compared to elite germplasm (modern varieties), but their genetic variability may allow them to cope with environmental stress and to be more resilient to climate change. Thereby, they became a potential source of favorable alleles to improve grain yield or pest resistance, and to give other favorable agronomic traits of new varieties [23,24].

Recent breeding programs studied and assessed genetic variability or different germplasm panels using different research approaches. Morphological and agronomical markers have been considerably used [25,26], with variable reproducibility depending on environmental conditions. . Nevertheless, this has been overtaken with the use of molecular markers which guarantee the opportunity of studying wheat phenotypes, providing reproducible and environment-independent results [27]. Several DNA markers have been developed and largely used to assess genetic diversity in tetraploid wheats [28-31] but the high-density genome coverage provided in recent years by single nucleotide polymorphism (SNP) markers have made them the best choice for wheat genetic analysis [32].

A novel plant breeding approach relying on human selection acting on an heterogeneous population (i.e., CCPs) has started few years ago under the name of Evolutionary plant breeding (EB) and represents a valuable method for developing populations adaptable to different agricultural contexts [33,34]. Cultivation conditions can drive the selection of more adaptable genotypes, which present increased fitness [35-37]. After several years of cultivation and multiplication in the same area under isolated conditions, these populations may reach equilibrium with stable yields and the genetic diversity among such populations represents a resilient trait to climate and environmental stress [38].

In this study, we investigate the genetic diversity and population structure of a panel of 264 accessions from seven tetraploid *T turgidum* subspecies coming from different Mediterranean and European areas using the 35k Wheat Breeders' Axiom® SNP array. This work will prove to be a groundwork for the identification of the genotypes, characterised by wide genetic diversity, that will be phenotyped in future field evaluation tests and lab analysis for the identification of best lines to be used in a cross-breeding program for the selection of resilient and nutritional improved wheat CCPs.

2. Materials and Methods

- *Plant Material*

A large Tetraploid wheat germplasm panel of 264 accessions was assembled at the Department of Agriculture DAGRI of the University of Florence (Supplementary Table S1). The core collection was represented by seeds of 8 *Turgidum* subspecies –ssp. *carthlicum* (5), *dicoccoides* (3), *dicoccon* (28), *durum* (172), *paleocolchicum (3)*, *polonicum (13)*, *turanicum* (33) *turgidum* (7) collected from the USDA bank, Wageningen CGN Germplasm search (https://cgngenis.wur.nl/ZoekGewas.aspx?ID=rrjkhxid&Cropnumber=01) and Istituto di Granicoltura di Caltagirone (www.granicoltura.it). One *T. aestivum* variety –Bologna- was added to the panel as outgroup genotypes.

Seeds were sown in peat-based soil in single pots and maintained in a climatic chamber at 15–25 °C with a day-night cycle of 16 h light and 8 h dark. Six weeks after germination, leaf tissue (5-6 cm section of a true leaf) was harvested from plants, immediately frozen on liquid nitrogen and then stored at 0 °C prior to nucleic acid extraction. All plants were then transplanted in the field and grew until maturity in order to collect seeds for single seed lines constitution to be used in future fields studies.

- *DNA extraction and genotyping*

Frozen leaf tissues were ground into a TissueLyzer bead mill (Qiagen) previously dipping tissue and plastic adapter into liquid nitrogen to avoid sample warming. Genomic DNA was extracted from leaf powder using a standard cetyltrimethylammonium bromide (CTAB) protocol [55] and then treated with RNase-A (New England Biolabs UK Ltd., Hitchin, UK) according to the manufacturer's instructions. DNA was checked for quality and quantity by electrophoresis on 1% agarose gel and Qubit™ fluorimetric assay (Thermofisher), respectively. The 35K Axiom® Wheat breed Genotyping Arrays (Affymetrix, Santa Clara, US) was used to genotype 265 samples for 35,143 SNPs using the Affymetrix GeneTitan® system at Bristol Genomics Facility (Bristol, UK) according to the procedure described in *Axiom® 2.0 Assay Manual Workflow User Guide Rev3* (https://assets.thermofisher.com/TFS-Assets/LSG/manuals/702991_6-Axiom-2.0-96F-Man-WrkFlw-SPG.pdf). This array contains a range of probes that are located onchromosomes belonging to the A, B and D genomes [56]. Since in tetraploid wheat the D genome was lacking, the effective number of markers that can be investigated was lower and correspond to 24,240 SNPs. Allele calling was carried out using the Axiom Analysis suite software [57] and a variant call rate threshold of 92% was used instead of the default value (97%) to account for the great heterogeneity of the set analyzed [58]. The same software was used to evaluate the number of monomorphic and polymorphic SNP markers, the heterozygosity level and the types of nucleotide substitution for each accession. Monomorphic SNP markers and those with missing data points were excluded from analysis. SNP markers were then filtered for minimum allele frequency (MAF) greater than 1 % and failure rate lower than 20%.

- *Statistical Analysis*

Data obtained from SNP genotyping of each accession were used to investigate levels and patterns of genetic diversity among them.. The Tamura-Nei method [59] for genetic distance evaluation, was applied to obtain a matrix of pairwise distances among accessions. and An unrooted Bayesian tree was computed applying the Neighbor Join algorithms [60] implemented in the "ape" 3.1 package of the R software [61].
To have a clear picture of the genetic structure of tetraploids wheat genotypes, we applied the Bayesian model-based clustering algorithm implemented in the STRUCTURE software version 2.3.4 [62]. An Admixtured and shared allele frequencies model was used to determine the number of clusters (K) assumed in the range between two and fifteen with five replicate runs for each assumed group. For each run, the initial burn-in period was set to 10.000 with 10.000 MCMC (Markov chain Monte Carlo) iterations, with no prior information on the origin of individuals. The best fit for the number of clusters, K, was determined using the Evanno method [63] as implemented in the program STRUCTURE HARVESTER [64]. Structure results were then elaborated with the R package "pophelper" in order to align cluster assignments across replicate analyses and produce visual representations of the cluster assignments. Discriminant analysis of principal components (DAPC) was used to infer the number of clusters of genetically related individuals [65], using the "adegenet" package in R-project [66]. DAPC first step was the data transformation using principal component analysis (PCA) while the second step was the discriminant analysis performing on the retained principal components (PCs). Groups were identified using k-means, a clustering algorithm which finds a given number (k) of groups maximizing the variation between them. To identify the optimal number of clusters, k-means was run sequentially with increasing values of k, and different clustering solutions were compared using Bayesian Information Criterion (BIC). The optimal clustering solution should represent to the lowest BIC [67].

3. Results

After SNP dataset filtering, 21,051 SNP markers were identified and used in the statistical analysis for the evaluation of the genetic diversity of the 264 tetraploid wheat accessions. The genetic relationships in the panel were assessed through three different approaches -Neighbor joining tree, discriminant analysis of principal components (DAPC) and STRUCTURE software- in order to better detail and define the genetic relationship variability among tetraploid accessions.

The Bayesian tree obtained by applying the Neighbor-joining algorithms revealed groups in the population that highly agree with the subspecies classification and origin (Figure 1). Most of the *T. turgidum* ssp. *durum* (yellow) were placed in a big clade together, with modern varieties that appeared separated from the other accessions. Landraces and old varieties were distributed in branches close together mostly according to their geographical origin as the Syrian and Sicilian accessions. Two other clusters were identified, respectively consisting of *T. turgidum* spp. *dicoccon* (orange) and *T. turgidum* ssp. *turgidum* (blue) while *T. turgidum* spp. *turanicum* (brown) clustered into two groups separated by the set of *T. turgidum* ssp. *polonicum* accessions (grey). The two *T. turgidum* ssp. *paleocolchicum* accessions (light blue) and their cross seemed to be close, while the few accessions belonging to *T. turgidum carthlicum* and *dicoccoides* ssp. appeared to be spread within the tree branches.

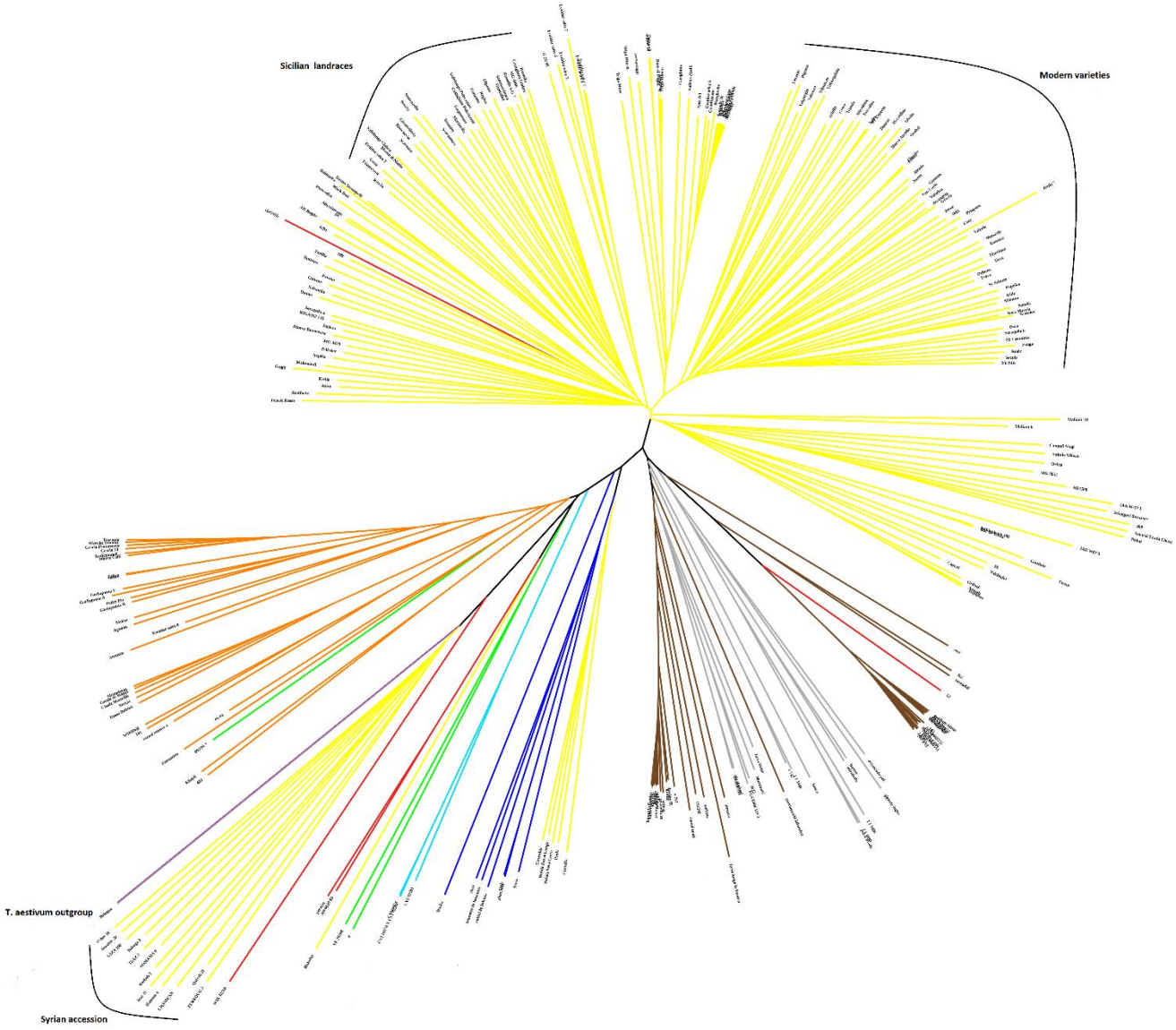

Figure 1. Bayesian tree of 264 tetraploid wheat genotypes based on SNP markers genetic and colored according to subspecies classification. Branches colours: yellow for *T. turgidum* ssp. *durum*, orange for *T. turgidum* ssp. *dicoccon*, brown for *T. turgidum* ssp. *turanicum*, grey for *T. turgidum* ssp. *polonicum*, blue for *T. turgidum* ssp. *turgidum*, pale blue for *T.*

turgidum ssp. *paleocolchicum*, red for *T. turgidum* ssp. *carthlicum*,green for *T.turgidum dicoccoides*,violet for *T. aestivum* outgroup accession.

Wheat genotypes arrangement obtained with the Bayesian tree was successively confirmed by the discriminant analysis of principal components (DAPC) results (Figure 2). Seven clusters (Figure 3) were detected in coincidence with the lowest Bayesian information criterion (BIC) value (Figure S1) and 100 PCs (80% of variance conserved) of PCA were retained. As reported in Figure 2, the Syrian *T. turgidum* spp. *durum* wheats pool in group 1 and clustered separately in the genetic tree. Most of the old varieties and landraces of the same subspecies were collected in group 2 while group 3 was approximately formed by the half of *T. turgidum* spp. *turanicum* accessions which belong to the same genetic cluster in the tree. The remaining genotypes of this last ssp. grouped together with *T. turgidum* spp. *polonicum* wheats which also cluster in group 5. Group 6 was entirely composed by *T. turgidum* ssp. *diccocon* accessions while group 7 identified the modern varieties of *T. turgidum* ssp. *durum*.

Moreover, both with the Bayesian tree and with the DAPC analysis, largely agreed with the accession's geographic origins. In particular, Syrian (cluster 1), French (cluster 7), Moroccan (cluster 4), Italian and Algerian (cluster 2) wheats, almost entirely pooled in the same cluster. Iranian (clusters 2 and 3), Portugal and American (clusters 4 and 5) accessions where equally divided in two clusters.

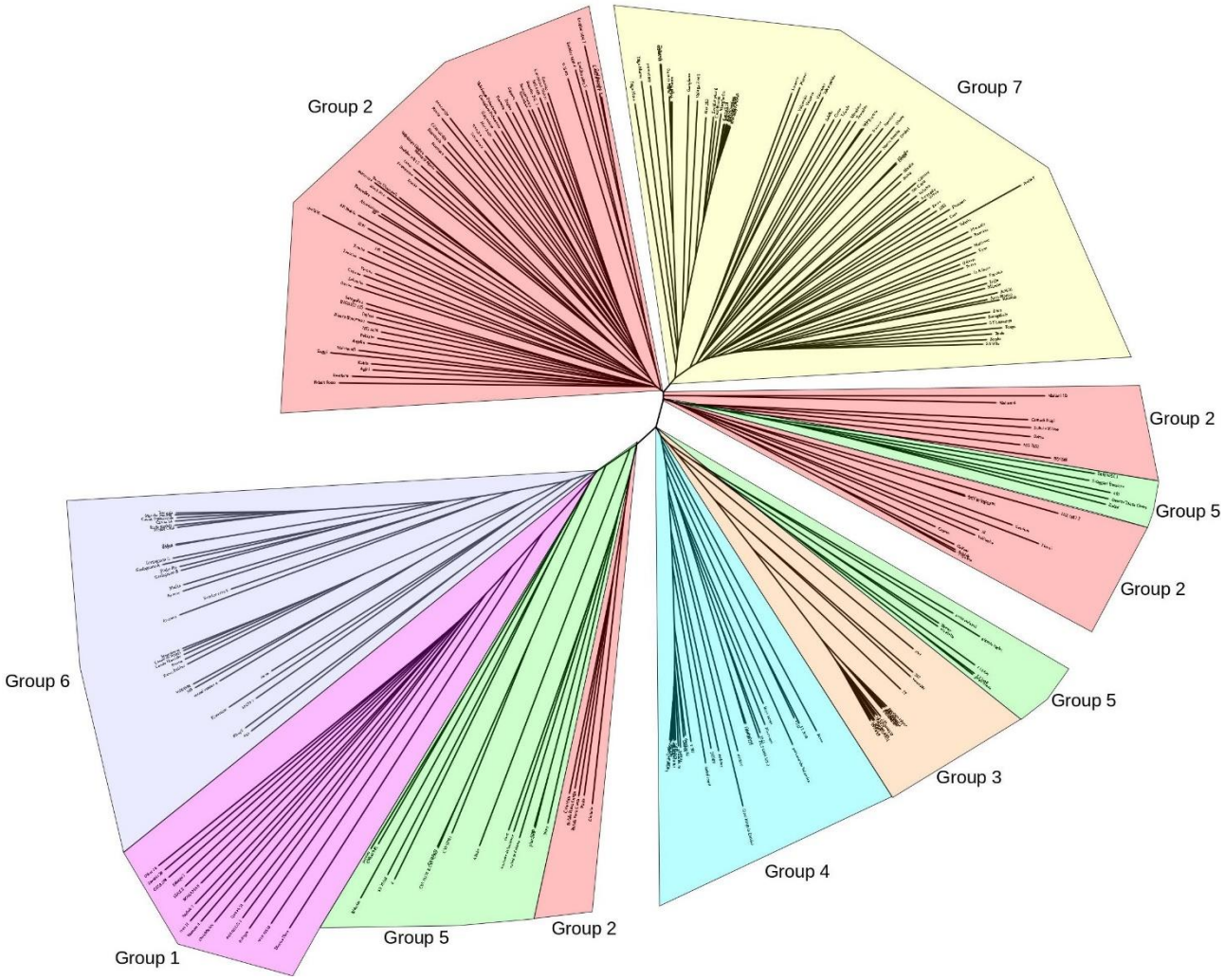

Figure 2. Phylogenetic tree of 264 tetraploid wheat genotypes based on SNP markers genetic and colored according to DAPC clusterization

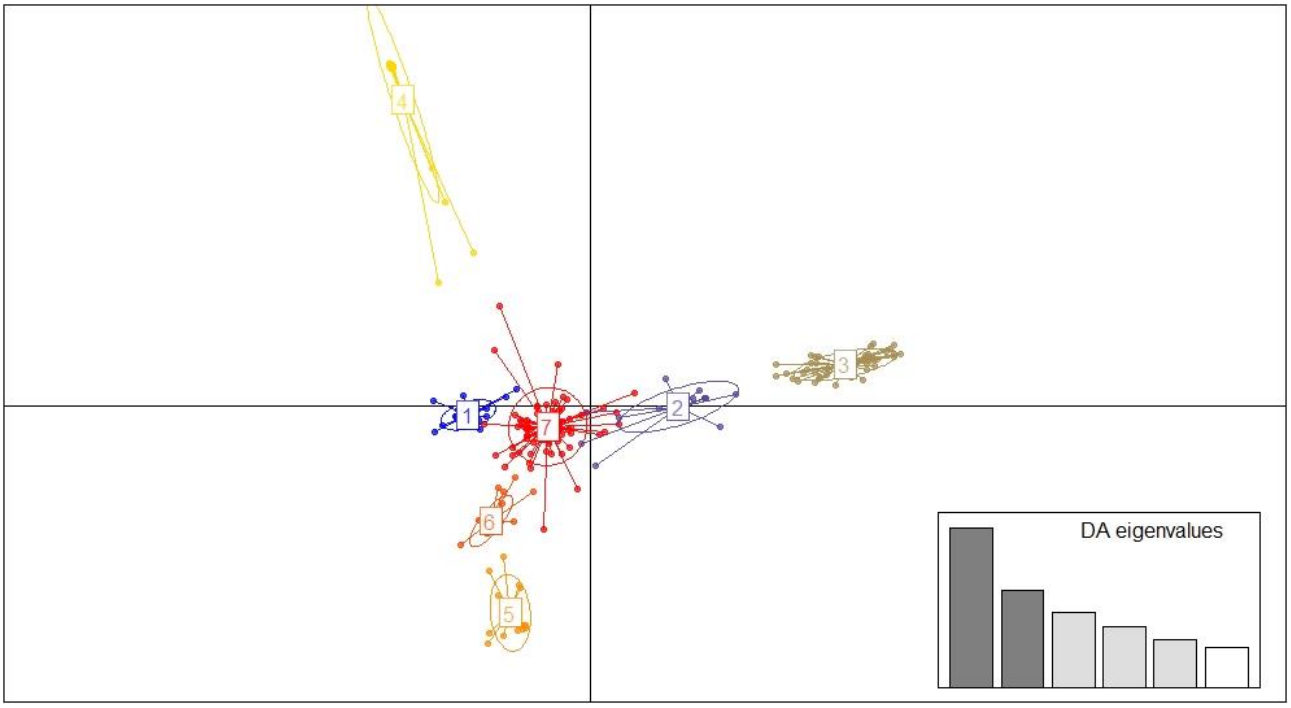

Figure 3. Discriminant analysis of principal components (DAPC) for 264 accessions of Triticum turgidum L. used for the analysis. The first two Linear Discriminants (LD) are represented by the axes. Each circle represents a cluster and each dot represents one accession. Numbers represent the different subpopulations identified by DAPC analysis.

The optimum number of sub-populations K, estimated using the STRUCTURE software (Figure 4) and according to the Evanno method results was was 7 (K = 7) . This indicated the presence of seven sub-populations, as previously found by Bayesian tree and DAPC analysis, although characterized mostly by different accessions.

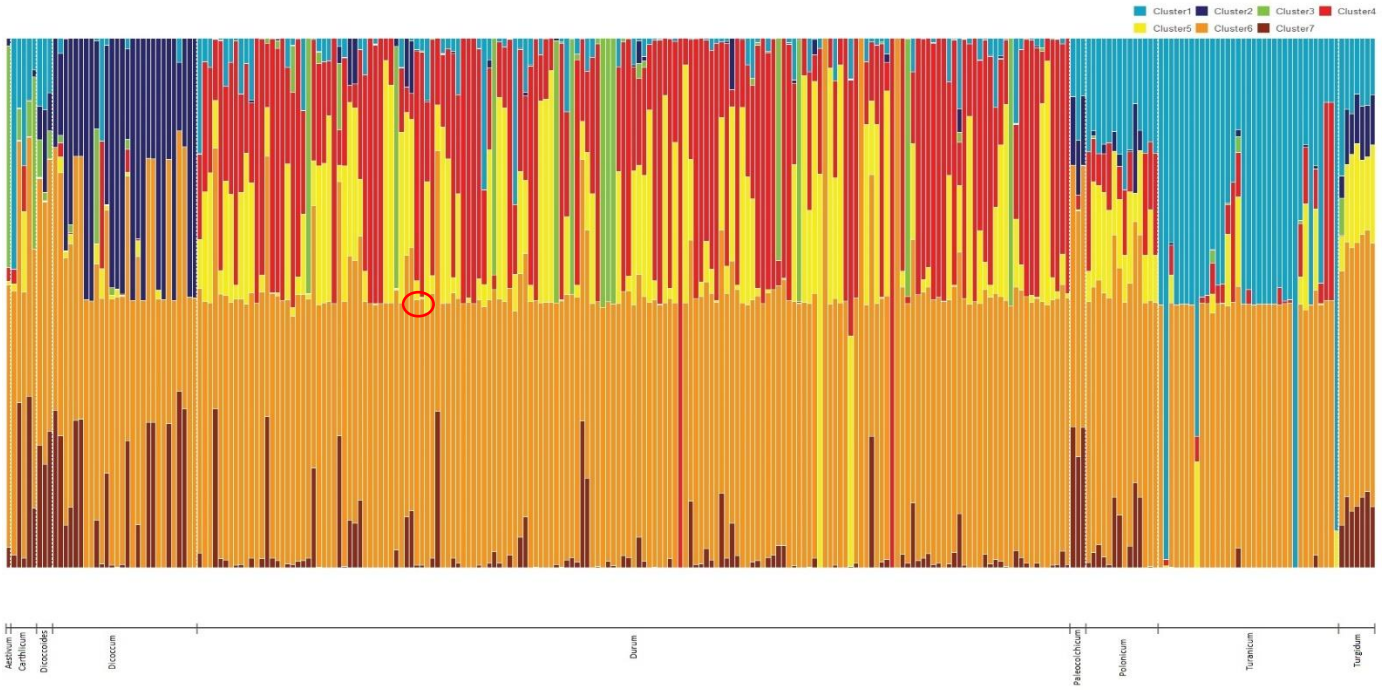

Figure 4. Diversity in admixture analysis by STRUCTURE among 264 tetraploid wheat accessions. Each individual is represented by a horizontal line. Color codes follow the number of clusters while the bar line under the graph represents the subspecies groups plus the outgroup genotypes (T. aestivum).

4. Discussion

Genetic diversity represents the base for crop improvement, providing plant breeders with germplasm necessary to develop cultivars with adaptive traits and quality characteristics [39]. To better target their crossing schemes, the genetic structure and variability of 264 tetraploid wheats accessions was assessed.
Clustering done with Bayesian tree and clusters obtained with DAPC revealed a clear classification of genotypes in accordance with their geographical origin, strengthening the previous studies of phylogenetic relationships of cultivated wheats and their wild relatives [40, 41].
Concerning *T. turgidum* ssp. *durum* accessions, which represented the largest number of genotypes in the panel, the first and second geographical origin centers –Syria and Ethiopia- of the subspecies [42, 43] appeared to be clearly identified, respectively in clusters 1 and 2. This result agreed with Kabbaj et al. [44] molecular assessment of a durum wheat collection of cultivars. More interestingly, the Bayesian tree highlighted the proximity between North African (Morocco, Algeria and Tunisia) and Italian germplasm: this could be linked to the geographical expansion of Romans during the Imperial Period and the consecutive wheat genotypes introduction and cultivation in the African continent, as suggested by Rickman [45].
In addition, the disposition of the accessions "Ciceredda", "Bufala rossa lunga", "Bufala nera corta" and "Paola" inside the Bayesian tree deserve attention: although they belong to cluster 2, which group almost all the other *T. turgidum* ssp. durum genotypes, they were gathered in a distant cluster between *T. turgidum turgidum* and *polonicum* ssp. This could due to a taxonomic problem, traceable thanks to De Cillis work [46] which classified these accessions under *T. turgidum turgidum* ssp. *turgidum* and could explain the proximity to this subspecies.
Finally, another relevant observation on *T. turgidum* ssp. *durum* accessions arrangement concerns the low genetic variability detected in the Italian modern varieties, differently from landraces and old varieties. Through the second half of the 20th century, national breeding programs, aimed at wheat yield increasing, started to establish new varieties characterized by low size, limited sprouting, reduced leaf area and crop cycle [47]. Due to the genetic improvement only, De Vita et al. [48] confirmed in their work a 44% increase in productivity for the main varieties of durum wheat grown in Italy during the 20th century but, on the other hand, this resulted in pure line selection and the development of varieties with low genetic variability [49]. Our study reflects this strong selection activity, gathering in the same cluster (Figure 2) and along neighbor branches Italian modern varieties and consequently highlighting a genetic homogeneity.
Contrary, the ssp. *dicoccon* showed the highest genetic variability, as Laidò [50] et al. verified in their research, confirming the powerful source of genes of this wild germplasm.
Today, the unpredictable climate, characterized by irregular rainfall and long dry periods, results in a rather unstable crop production. Under marginal environments, landraces and old varieties showed higher stability in low-input agriculture [51,52], thus, they could represent valuable genetic resources for breeders in order to develop new cultivars with specific qualitative traits as resistance to biotic and abiotic stress and nutritional ones. The development of successful CCP populations for low-input farming systems should select parental lines from local landraces or wild relatives in order to provide them with the ability to resist biotic and environmental stress and efficiently use organic nitrogen [53, 54] With this aim, our results showed the genetic diversity among accessions belonging to seven tetraploid wheat subspecies and identified the correct numbers of genotypes that well explained all the genetic variability screened.

Conclusions

Genetic diversity of domesticated wheat accessions has been significantly reduced compared to that of their wild progenitors, through a prolonged selection process for those phenotypic traits which better satisfied human needs. On the contrary, landraces genetic variability represents a precious source of valuable agronomic traits which could be used for interspecific hybridization and for introgression of genes or/and alleles into cultivated species. In our work, the genetic diversity and the population structure of 264 tetraploid

wheats were investigated in order to understand the genetic relationships between domesticated wheats and their close wild relatives. The results obatained from this research could be used in future phenotyping studies in both field and laboratory tests to select the best lines to be intercrossed for the creation of durum wheat improved and resilient CCP populations adapted to Mediterranean areas.

Supplementary materials: Figure S1: Statistical determination of the optimum number of clusters by discriminant analysis of principal components (DAPC), Table S1: List of wheat accessions used in the experiment.

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