





High-throughput genotyping, morphological and

quality traits to assess genetic diversity of wheat landraces from Sicily

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- Abstract: During the XX Century, the widespread of modern wheat cultivars drastically reduced the cultivation of ancient landraces, nowadays confined in niche cultivation areas. Several durum wheat landraces are still cultivated in Sicily (Italy), because of a high adaptability to extreme Mediterranean environments. Detailed knowledge about genetic diversity on this germplasm could lay the basis for an efficient management of genetic resources in a breeding perspective, due to their tolerance against biotic and abiotic stresses compared to modern variety. The aim of the present study was to characterize a collection of Sicilian durum wheat landraces using a genotyping array, together with agro-morphological, phenological and qualitative traits, using the modern cv. Simeto, Claudio and the hexaploid landrace, Cuccitta, as outgroups. Cluster analysis and Principal Coordinates Analysis (PCoA) allowed us to identify 4 main clusters across the analyzed germplasm, among which a private cluster included modern and commercial varieties. Likewise, STRUCTURE analysis was able to distinct the ancient varieties from the others, grouping the entries in seven cryptic genetic clusters. Furthermore, a PCA was able to separate the modern testers from ancient germplasm. This approach was useful to classify and evaluate Sicilian ancient wheat germplasm, supporting their safeguard and providing a genetic fingerprint useful to avoid commercial frauds and sustain the economic profits of farmers resorting to cultivation of landraces.

Keywords: wheat landraces, genetic diversity, Single Nucleotide Polymorphism, morphological and quality traits.

1. Introduction

Wheat (*Triticum* spp.) is the largest primary commodity and one of the oldest and most important cereal crops, being grown on more land area than any other crop worldwide. Durum wheat (*Triticum turgidum* L. subsp. *durum* Desf.) is the second most important *Triticum* species, next to common wheat (*Triticum aestivum* L.), and is mainly used to produce semolina and dried pasta. The European Union (EU) holds the largest share in world durum wheat harvested production (8,956.58 thousand tonnes), Italy being the main EU producer with 4,303.10 thousand tonnes (EUROSTAT, 2018; https://ec.europa.eu/eurostat/web/agriculture/data/database). Durum wheat is a crop of

primary importance for the Mediterranean region and is mainly cultivated under rainfed conditions, resulting in major yield variations due to erraticism of rainfalls. Outside of the Mediterranean region, durum wheat is cultivated in Northern America (Canada, U.S.A.) and Central America (Mexico), as well as in smaller growing areas of Russia, Australia, Argentina and Ethiopia.

Durum wheat is a member of the *Gramineae*, included in the *Triticeae* tribe belongs to the *Triticum* genus. Based on cytological and molecular analyses, *Triticum turgidum* is believed originated from a hybridization between *Triticum urartu* (AA genome, n=7) and an unknown diploid species (BB genome, n=7) close related to *Aegilops speltoides* [1]. Thus, durum wheat is a self-pollinated allotetraploid cereal (harboring two genomes with genomic formula: AABB) with 28 chromosomes (2n=4x=28). All *Triticum* species are native to the *Fertile Crescent* of the Near East. In particular, Jordan River valley and Levant Corridor, a quite narrow strip between the between the Mediterranean Sea to the northwest and deserts to the southeast, localize the origin of domesticated tetraploid wheats, including durum wheat. Wheat cultivation was spread westwards to Europe (Italy, France and Spain) and North Africa during the Neolithic period, through Anatolia and Greece. During the ancient Greek colonization, wheat was introduced in Sicily, but only under Roman domination in the third century BC Sicily became important as the *Granary of Rome*.

At the beginning of last century, durum wheat breeding was pioneered in Italy, when durum and common wheat started to differ more clearly [2]. Many tetraploid wheat landraces were already present in Italy when different exotic landraces were introduced and included into breeding programs [3, 4]. In the first 20 years of the XX century, Nazareno Strampelli research activity led to firstly develop cultivars based on the selection of available diversity, so that key pivotal results were achieved through the development of pure lines breeding programs starting from Southern Italy local races. Strampelli project was continued by many Italian breeders with the release of new varieties in the 50s-60s exploiting a large genetic variability [5]. These cultivars already harbored GA-insensitive dwarfing genes introgressed by North African wheat germplasm and the intensive activities of wheat breeding in Italy continued during the second half century at the Agricultural Experimental Stations and the Academies mainly to improve crop yield and quality with a particular emphasis to traits related to plant adaptation.

The spread of modern wheat cultivars drastically reduced the cultivation of the "old landraces", confined in niche areas of cultivation, the maintenance of which has been entrust to public research institutions and custodian farmers. Nowadays, this germplasm represents an important genetic resource because of ancient selections conducted by farmers with the interaction with local climatic conditions as reported by De Cillis [6]. The preservation of "historical cultivars" and/or landraces could be important to avoid genetic erosion, fostering their employment in new breeding programs [7]. Indeed, a higher tolerance of ancient landraces against biotic and abiotic stresses compared to modern varieties has been demonstrated [8, 9]. Nowadays attention is given to cereals nutritional values and health effects and the terms "ancient grains", "historical varieties", "modern varieties" have become common in the scientific community and among consumers, covering a broad subject

area including the identification of varieties. Growing consumer interest in healthy food production stimulated research about the phytochemical content of ancient and modern durum wheat varieties [10], and this trend led to rediscovery and reutilize durum wheat landraces, including those in Sicily [11].

Knowledge on genetic diversity of durum wheat landraces should be the starting point for their utilization and conservation management, while this genetic diversity needs to be well described and measured. Wheat genetic diversity was largely evaluated using morphological and phenological descriptors established by the International Plant Genetic Resources Institute (IPGRI) [12] by the International Union for the Protection of New Varieties of Plants (UPOV) [13], and including the assess of seed storage proteins variation. Nowadays, molecular markers represent a powerful tool to analyze the genetic diversity avoiding misclassifications related to environment effects. These assessments could also help us to maintain the greatest diversity with the least reappearance of samples for conservation. Among molecular markers, single nucleotide polymorphisms (SNP), the most common polymorphism among individuals of any species [14, 15], became one of the most powerful tools in molecular biology [16]. SNP are point mutations that result in single base-pair divergence among DNA sequences and can be present in both coding and noncoding regions of the genome. The advent of high-throughput Next Generation Sequencing (NGS) technologies allowed to get large-scale SNP identification for plant species and to develop efficiency SNP genotyping platforms for association studies [17, 18] and genetic characterization also in wheat [19-26].

Among Mediterranean regions, Sicily is among the richest in wheat landraces diversity. In Sicily, several durum wheat landraces were recently rediscovered and re-employed in cultivation, because of high adaptability to extreme Mediterranean environments also due to their genetic structure typical of a self-pollinated population (mixture of different pure lines). Anyhow, there are considerable uncertainties regarding the correspondence between the varieties descripted by Ugo De Cillis [6] and those currently available and cultivated. Misperception about denominations (different names of the same landrace in the different areas of cultivation) is recurrent and germplasm conservation techniques not always suitable helped to generate some confusion.

The *Experimental Sicilian Station for Durum Wheat (ESDW)* at Caltagirone (province of Catania, Italy), is the wheat research station responsible for the conservation of local varieties, breeding and research for wheat growers and processors in Sicily. The ESS bred and preserved over 200 accessions along the years including 48 local varieties of *Triticum durum, T. aestivum* and *T. turanicum*, previously collected and described thanks to the effort of De Cillis [6]. Anyhow, the germplasm collection is preserved in Caltagirone, maintained and seed multiplied in purity from almost 15 years. Some landraces are still cultivated in Sicily while the others represent unique genetic resources to be exploited in future breeding program. In the panel, commercial varieties largely spread in the last century in Sicilian wheat growing areas were included, among which "Cappelli" registered in the *Italian national register* of *varieties* in 1915 and still widely cultivated, and "Trinakria", registered in 1970 but currently phased out.

The aim of this study was to evaluate the genetic diversity in a wheat collection from the ESDW throughout a SNP array, also conducting agro-morphological, phenological and qualitative traits analysis. The evaluation and classification of wheat landraces will allow to well preserve these genetic resources, to promote their sustainable use, eventually providing a reproducible genetic fingerprint useful to avoid commercial frauds and to sustain the economic profits of the farmers.

2. Results and Discussion

2.1. Morphological and grain quality traits

Durum wheat collection effectively preserved and documented at the *ESDW* were characterized for different morphological, phenological and grain quality traits. A heatmap was generated for a simplified representation of morphological traits distribution (Figure 1).





Figure 1. Heatmap of morphological traits measured on Sicilian wheat germplasm (27 landraces; in italic), 2 historical varieties (Cappelli and Trinakria; in bold) and 1 bread wheat landrace (Cuccitta; underlined). Green and red colors represent reduced and augmented representation levels, respectively. Hierarchical clustering of samples (cluster 1 - 5) and traits are also shown. All the traits, coded as morpho 1 to morpho 27, are described in Table S1.

Overall, these traits allowed us to group the germplasm in five main clusters, with 50% of landraces/cultivars belonging to cluster 1, and 5, 4, 3 and 3 genotypes belonged clusters 2, 3, 4 and 5, respectively. Interestingly, ten traits showing high variability among samples determined their clustering: six traits (morpho 2, morpho 11, morpho 19, morpho 23, morpho 24 and morpho 27; Table S1) were mainly due to the genotypic variation; while three traits were highly influenced by environment (morpho 3, morpho 4 and morpho 17; Table S1). In addition, flag leaves (morpho 2)

plays a very important role in grain filling and yield together with ear emergence (morpho 3), ear cross shape (morpho 23) and density (morpho 24).

The reduction of plant height, together with higher grain yield and early maturation, were the main breeders aims in the last century. Our historical Sicilian wheat collection, here investigated, showed mostly a growth *erect* or *semi erect* (data not shown) and plant height ranged from 95 and 135 cm. Nineteen % of the landraces showed a plant height below 120 cm (Table S1). Based on the data collected, the time of ear emergence, counted as days from sowing to flowering, ranged from *early* to *medium* in more than 70% of the collection (Table S1). Among landraces, it is possible to recognize genotypes belonging to the *'syriacum typicum'*, a group utilized to select and release shorter and earlier-flowering new cultivars in Italy during 1920–1950 [27]. These results support previous reports [28], confirming the key role of these genetic resources for a potential re-introduction of historical durum wheat landraces in cultivation.

ANOVA of grain and wholemeal flour qualitive traits of wheat genotypes are reported in Table 2. In durum wheat that have not undergone weathering, test weight (TW) is an excellent predictor of semolina milling potential. Indeed, this parameter indicates the specific seed weight per unit volume depending on filling degree of the seed and is used to indicate the likely milling performance of wheat and considered a more reliable predictor of flour/semolina yield [29, 30]. Here, there are no significant differences between the highest value recorded for *cv*. Claudio and those measured in two landraces (val2gl and tri2). The same for other five landraces (urr1, tre2, trin, sam3 and bivc) when compared to the value recorded for *cv*. Simeto. The thousand-kernel-weight (TKW) ranged from 33.5 to 59.2 g recorded in "tim" and "val2gl", respectively. Interestingly, thirteen out of twenty-seven landraces recorded a significant TKW higher values as compared to *cv*. Claudio, further seven landraces showed higher TKW values also than modern *cv*. Simeto. These performances might be due to earlier flowering of modern wheat cultivars as previously reported [31]. These results indicate that durum wheat landraces from Sicily might possess a higher yield

potential than modern cultivars when cultivated under drought stress conditions.

Non-vitreous (starchy) kernels, opaque and softer, influence on the milling process, reduce to fines, decreasing semolina extract [32]. The incidence of starchy kernels, recorded on Sicilian wheat collection, ranged from 0 (sco4) to 96% (tri2), with landraces showing higher percentage compared to modern cultivars as reported in Gallo et al. [33].

Wholemeal flour quality traits, protein and gluten contents are the most important features useful for characterizing durum wheat landraces/cultivars. Indeed, it is well documented that protein content and endosperm storage protein composition have a decisive impact on wheat processing quality [34, 35]. High protein level on semolina will usually yield a product with uniform particle size with a minimum number of starchy particles although it has been shown that other traits, like such specific y-gliadins and gluten viscoelasticity, together with vitreousness and yellow semolina colour, play a pivotal role in quality, processed wheat products [36, 37].

ID	Test weight (kg/hL)	Thousand kernels weight (g)	Starchy kernels (%)	Protein content (% d.m.)	Wet gluten content (%)	Dry gluten content (%)	Water binding in wet gluten	Gluten index (0-100)	B
bia1	79.2±0.14 ^e	41.9±0.14 ^q	2.5 ± 0.71^{ln}	15.5±0.07 ^a	36.00±0.57 ^{eg}	11.10±0.2 ^{eg}	24.90±0.28e	48.60±0.81 ^J	1
bd3	77.6 ± 0.14^{gh}	39.2±0.14 ^r	5.5±0.71 ^{il}	17.1 ± 0.00^{a}	42.45±0.64 ^b	14.70±0.28ª	27.75±0.35°	56.65±0.65 ^{eg}	14
bivc	$80.2{\pm}0.28^{d}$	54.9 ± 0.07^{d}	43.5±2.12 ^{cd}	14.1±0.07 ^e	32.25 ± 0.92^{il}	9.65±0.21 ^{jk}	22.60±0.71 ^{gh}	36.10 ± 1.82^{mn}	1
bb2	71.8±0.14 ^q	45.3±0.14 ^{no}	15.5 ± 2.12^{f}	14.9 ± 0.07^{cd}	31.45 ± 0.92^{jl}	$10.10{\pm}0.28^{hj}$	21.35 ± 0.64^{gi}	$54.51{\pm}1.33^{\rm fh}$	16
bnc2	73.9±0.07 ^{no}	43.2±0.14 ^p	9.5 ± 2.12^{gi}	14.1±0.00 ^e	$34.40{\pm}1.13^{gi}$	$9.95{\pm}0.35^{hj}$	$24.45{\pm}1.48^{ef}$	$37.18 {\pm} 2.07^{mn}$	1
brc-b1	75.6 ± 0.14^{1}	49.2±0.21 ^{ij}	28.5±2.12e	14.1±0.07 ^e	42.25±0.50 ^b	11.80±0.14 ^{ce}	30.45 ± 0.35^{b}	48.40 ± 0.60^{j}	1
capp1	$81.4{\pm}0.07^{b}$	49.6±0.58 ⁱ	$9.0{\pm}1.41^{gi}$	15.1 ± 0.00^{ab}	38.10±0.85 ce	$12.00{\pm}0.14^{cde}$	26.10±0.71 ^{cde}	19.67±1.79 ^q	1
cas1pu	$76.8 {\pm} 0.07^{ik}$	49.7±0.21 ⁱ	$1.0{\pm}0.00^{no}$	$14.8{\pm}0.07^{d}$	$32.00{\pm}1.27^{il}$	$10.00{\pm}0.1^{hj}$	$22.00{\pm}1.13^{gi}$	29.01±2.82°p	1
cic1	$78.4{\pm}0.07^{\rm f}$	42.8±0.21 ^p	0.5±0.71°p	16.7 ± 0.00^{a}	40.10 ± 0.85^{bc}	12.55±0.21 ^{bc}	27.55±0.64 ^{ce}	$35.40{\pm}1.37^{n}$	1
cla	$82.6{\pm}0.28^{a}$	48.4 ± 0.35^{k}	11 ± 1.41^{fh}	13.95±0.07 ^e	30.79 ± 0.72^{jl}	$9.85{\pm}0.78^{\rm kl}$	$21.74{\pm}0.65^{gi}$	83.07 ± 1.39^{b}	1′
cot1	$77.8{\pm}0.00^{gh}$	$48.8{\pm}0.28^{jk}$	46.5±3.54°	14.0±0.07 ^e	$32.09\pm\!\!1.56^{\rm hk}$	$9.85{\pm}0.78^{\ ik}$	$23.05{\pm}0.78^{fg}$	59.53±1.91°	1
cuc1	73.6±0.14°p	47.7 ± 0.21^{1}	$11.5 \pm 2.12^{\text{fh}}$	$15.4{\pm}0.00^{a}$	$33.55{\pm}0.78^{gj}$	$11.10{\pm}0.42^{eg}$	$22.45{\pm}0.35^{gi}$	$66.31{\pm}0.78^{d}$	1
fsa1	$78.5{\pm}0.00^{\rm f}$	$50.6{\pm}0.28^{h}$	2.5 ± 0.71^{ln}	$12.5{\pm}0.07^{h}$	$20.70{\pm}0.42^{n}$	$7.00{\pm}0.42^{n}$	13.70 ± 0.00^{1}	$18.82{\pm}1.66^{q}$	15
gig1	74.1 ± 0.00^{no}	47.5 ± 0.28^{lm}	$1.0{\pm}0.00^{no}$	$16.2{\pm}0.00^{a}$	40.50 ± 0.99^{bc}	13.15 ± 0.07^{b}	27.35±0.92 ^{ce}	$44.43{\pm}1.36^{k}$	1
gio1	75.5 ± 0.21^{lm}	53.5±0.14 ^e	1.5 ± 0.71^{mo}	14.7 ± 0.07^{d}	30.20±0.71 ¹	9.65 ± 0.21^{jk}	20.55 ± 0.49^{1}	26.47 ± 1.72^{p}	1
giu1	76.5 ± 0.21^{jk}	$45.0\pm0.07^{\circ}$	2.5 ± 0.71^{ln}	$15.5{\pm}0.07^{a}$	$35.25{\pm}0.64^{\rm fh}$	$10.80{\pm}0.28^{\rm fh}$	$24.45{\pm}0.35^{ef}$	$34.17{\pm}1.19^{n}$	10
ing2	77.2 ± 0.14^{hi}	$49.3{\pm}0.07^{ij}$	$3.5{\pm}0.7^{jn}$	16.1 ± 0.00^{a}	39.45 ± 0.35 ^{cd}	$12.30{\pm}0.14^{bd}$	27.15±0.21 ^{cd}	$43.47{\pm}0.51^{k}$	1
mar2	$75.4{\pm}0.07^{\rm lm}$	51.5 ± 0.07^{g}	$14.0{\pm}1.4^{\rm fg}$	12.9±0.00g	20.20 ± 1.70^{n}	7.75±0.35 ^{no}	$12.45{\pm}1.34^{\rm lm}$	78.64±1.79°	1
mla1	$75.1{\pm}0.14^{m}$	46.9±0.14 ^m	$7.0{\pm}1.4^{\rm hij}$	12.9±0.00 ^g	18.05±1.061°	6.75±0.35 ^m	11.30±0.71 ^{mn}	91.12±0.52ª	1
pao2	$76.9{\pm}0.07^{ik}$	57.4±0.14 ^b	66.5±3.54 ^b	15.0 ± 0.00^{bc}	45.40±0.71 ^a	12.50 ± 0.42^{bc}	32.90±0.28ª	$50.88{\pm}0.77^{ij}$	14
rea4	73.7 ± 0.14^{no}	$41.4{\pm}0.42^{q}$	$9.0{\pm}1.41^{gi}$	15.0 ± 0.07^{bc}	$31.75{\pm}0.35^{jl}$	$10.80{\pm}0.42^{\rm fh}$	$20.95{\pm}0.07^{\rm hi}$	$52.12{\pm}0.53^{hi}$	1
reg1	$76.4{\pm}0.21^{k}$	56.6±0.28°	$4.0{\pm}1.41^{\text{jm}}$	16.3 ± 0.00^{a}	$39.00{\pm}0.28^{cd}$	11.35 ± 0.07^{dg}	27.65±0.21°	$42.31{\pm}0.42^{kl}$	1
rus1	77.1 ± 0.07^{ik}	57.4±0.21 ^b	$10{\pm}1.41^{\rm fi}$	13.3 ± 0.07^{f}	$24.90 \pm 0.28^{\mathrm{m}}$	$8.80{\pm}0.28^{1}$	$16.10{\pm}0.00^{k}$	$57.83{\pm}0.48^{\text{ef}}$	1
sam3	$80.2{\pm}0.14^{d}$	$51.2{\pm}0.2^{gh}$	$6.5{\pm}0.71^{hk}$	14.9±0.07 ^{ce}	38.80 ± 0.57 ^{cd}	11.65 ± 0.07^{cf}	27.15±0.49 ^{ce}	48.45 ± 0.75^{j}	1
sco4	73.3 ± 0.21^{p}	$44.7 \pm 0.14^{\circ}$	$0.0{\pm}0.00^{p}$	16.7 ± 0.07^{a}	38.45 ± 0.64 ^{ce}	12.10 ± 0.14^{cd}	26.35±0.78 ^{ce}	$54.22{\pm}0.76^{gi}$	1
sim	79.1±0.14 ^e	52.3 ± 0.28^{f}	$3.0{\pm}1.41^{kn}$	$14.65 {\pm} 0.07^{d}$	31.63 ± 0.56^{jl}	9,30±0.14 ^{j1}	$22.33{\pm}0.42^{gi}$	$86.30{\pm}0.25^{b}$	1
tim1	$75.3{\pm}0.28^{\rm lm}$	33.5 ± 0.00^{s}	$9.0{\pm}1.41^{gi}$	13.5 ± 0.14^{f}	$26.95 \pm 0.50^{\mathrm{m}}$	8.55 ± 0.07^{1}	18.40 ± 0.42^{j}	30.60±1.27°	1
tre2	80.3 ± 0.14^{cd}	47.5 ± 0.21^{lm}	26.5±2.12 °	$11.9{\pm}0.07^{n}$	14.00 ± 0.28^{p}	5.30 ± 0.14^{p}	8.70±0.14°	67.14 ± 0.66^{d}	1:
trin	$80.3 {\pm} 0.14^{cd}$	56.5±0.21°	$7.0{\pm}2.83^{hj}$	$14.80{\pm}0.00^{ij}$	32.35 ± 1.34^{il}	$10.70{\pm}0.42^{gi}$	$21.65{\pm}0.92^{gi}$	$83.60{\pm}0.68^{b}$	10
tri2	$82.3{\pm}0.07^{a}$	39.3 ± 0.07 r	96.0±1.41 a	$8.5{\pm}0.04^{k}$	15.00±0.14 ^p	$5.40{\pm}0.14^{p}$	9.60±0.28 ^{no}	77.33±0.21°	1
urr1	$80.7 \pm 0.00^{\circ}$	45.7±0.14 ⁿ	$38.0{\pm}1.41^{d}$	11.5 ± 0.07^{j}	18.85 ± 0.07 no	$6.20{\pm}0.00^{\circ}$	12.65 ± 0.07^{lm}	$38.99{\pm}0.23^{\rm lm}$	1
val2gl	$82.3{\pm}0.28^{a}$	59.2±0.21 ^a	$7.0{\pm}1.41^{hj}$	$15.7{\pm}0.00^{a}$	$37.15{\pm}0.07^{df}$	$11.50{\pm}0.14^{dg}$	25.65 ± 0.07^{de}	$34.05{\pm}0.13^{n}$	1

Table 2.	One-way	ANOVA	for grain	and wh	olemea	l flour	qualitative	parameters	of Sicilian v	vheat
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Data are expressed as the mean value \pm standard errors of means different letters denote significant differences between treatments at the at P \leq 0.001 according to the Tukey's test

Frequently, modern durum wheat cultivars show lower grain protein content compared to the older ones [38-40], because of wheat breeding programs mainly focused on increasing grain yield [41]. Our result seems to confirm these previously reports. Indeed, significant higher protein content (>16.0%) was observed in six Sicilian landraces (bd3, sco4, reg1, cic1, gig1 and ing2) compared to modern cultivars (<15%), whereas the lowest value (8.5%) was found in tri2, due to the high percentage of starchy kernels (Table 2).

The values of wet and dry gluten content, important for pasta industry, showed a comparable trend (r²=0.97) with protein content and a wide variability as already reported [42, 43]. The historical *cv*. Capp1, recently registered again in the Italian National Register of Varieties (INRV) for its reintroduction in wheat cultivation areas of Italy, showed a very high value of wet gluten content (38.1%). As regard to gluten index, often correlated to gluten quality, most of the ancient wheat landraces showed low values, ranged from 18.8 (fsa1) to 59.5 (cot1) (Table 2; Figure S1). By contrast, "mla1" landrace showed a significant higher value (91.12) compared to testers (86.3 and 83.1 in Claudio and Simeto, respectively). These data confirmed the presence of a weaker gluten in Sicilian wheat landraces as previously reported [44-47], compared to modern cultivars [48-50].

Durum wheat grain color might affect the quality of the end products, depending by genetic traits, but also environment growing condition and the technological processes. The anthocyanins are a class of pigments, which characterize durum wheat aleurone or pericarp. The high-level of these pigments observed in many Sicilian landraces represents an important trait for breeding programs aimed at improving the nutritional value of grain and its end products [51]. The yellow index (b*) measured in semolina was significantly higher in the *cv*. Simeto (20.06), compared to those measured in the Sicilian landraces demonstrating the effect of selection on this important nutritional trait. The historical *cv*. Cappelli (capp1) together with two landraces, "fsa1" and "rea4" were characterized by high value of yellow index (b*) according to Digesù et al. [52]. As expected, the bread wheat landrace (cuc1) showed the significant lowest yellow index (8.74).

The Principal Component Analysis (PCA) performed on data recorded for all qualitative grain traits depicted an overview of wide differences among landraces (Figure 2). The first two components explained 60.1% of total variance, with the second ones able to discriminate cuc1 (*T. aestivum* L.) from the landraces belonging to *T. turgidum* L. *ssp. durum* and these last from historical and modern cultivars (Figure 2). Protein content and the parameters related to gluten showed a strong influence on both components (cos2=0.75), separating 50% of landraces (16) from the others. Finally, *T. aestivum* L. was characterized by a higher number of starchy kernels (cos2=0.75), strongly related to the first component (Figure 2). These evidences were confirmed by Pearson correlations, showing a positive correlation (p<0.05) among water binding in wet gluten, dry and wet gluten, with the higher positive correlation coefficients (0.96) for the last two components and a negative correlation (p<0.05) between starchy kernels and protein content (-0.64) (Figure S2). Likewise, red, brown and yellow index were positive correlated (p<0.05).



Figure 2. Principal Component Analysis (PCA) of qualitative traits. Based on their origin, samples were organized in 4 main groups: (1) *T. aestivum* L., (2) *T. turgidum* L. subsp. *durum* historical varieties (Cappelli and Trinakria,), (3) *T. turgidum* L. subsp. *durum* ancient landraces (27) and (4) *T. turgidum* L. subsp. *durum* modern varieties (Claudio and Simeto). Traits associated to samples discrimination were indicated in the plot, underlining their significance values (0.25<cos2<0.75). The medians for each group were also visualized.</p>

2.2. SNP analysis and genetic characterization

Genetic diversity can be evaluated by biochemical and morphological markers and/or the use of pedigree. Unfortunately, these approaches can be influenced by environment or can be erroneous and incomplete, causing frequently misclassifications among genotypes. By contrast, DNA markers allow the assessment of relatedness directly at the DNA level, thus molecular markers became necessary for identifying genetic variation among and within landraces/populations due to environmental effects on many traits with a polygenic control. Different molecular markers can be used for comparative genomic, phylogenetic relationships and diversity studies [53, 54]. Differently, next generation sequencing (NGS) led to set up different SNP panels successfully used for genetic diversity analyses also in wheat [21, 55]. The high-throughput wheat 90kSNP array was used to investigate the genetic relationships across the Sicilian ancient germplasm, using two modern cultivars (Claudio e Simeto) as references and Cuccitta, a bread wheat landrace, as outgroup. After SNP-dataset filtering, 5,594 loci (7%) did not amplify among all genotypes and 41,926 (51%) were monomorphic (Table 3). The final dataset resulted in 20,539 loci after removing the SNPs with a

number of NC (not-call) higher than 20%, among which 13,528 loci (66%) were polymorphic with the overall MAF value of 0.232 (Table 3).

Table 3. Summary statistics of genetic variation obtained by the wheat 90k SNP array in 27 landraces and 2 historical varieties (Cappelli and Trinakria) belonging to Sicilian wheat germplasm. The modern varieties Claudio and Simeto were also added as references, while hexaploid Cuccitta landrace (*T. aestivum* L.) was included as outgroup.

Parameters	Values
n [†]	32
Total number of loci	81,587
80 % failed loci	5,594
No. of monomorphic loci	41,926
No. of used loci	20,539
No. of polymorphic loci	13,528
MAF	0.232

⁺Sample size, MAF, minor allele frequency; H_e, expected heterozygosity; H_o, observed heterozygosity.

To investigate the genetic relationships among cultivars based on SNP-data, phylogenetic analysis and PCoA were carried out. Cluster analysis based on Nei (1978) genetic coefficient and the UPGMA algorithm generated a dendrogram underlining four main clusters across durum wheat germplasm (Figure 3). Two modern and the historical cultivars (Claudio and Simeto; Trinakria and Cappelli, respectively) grouped in Cluster A together with three landraces (brc-b1, mar2 andbd3), while the bread wheat landrace Cuccitta was the outgroup, as expected (Figure 3). Cluster B included 16 out of 27 durum wheat Sicilian landraces (60%), while cluster C and D grouped only 3 and 5 landraces, respectively. Nearly 80% of bootstrap values among cluster ranged from 99 to 100% for the most nodes avoiding any misclassifications (Figure 3).



Figure 3. Genetic relationships among ancient landraces and 2 historical varieties Cappelli (capp1) and Trinakria (trin), belonging to Sicilian wheat germplasm collection, obtained by using 13,528 polymorphic SNPs. Simeto (Sim), and Claudio (Cla) were utilized as testers, while hexaploid Cuccitta was added as outgroup. Dendrogram generated using the UPGMA method and Nei's distance.

PCoA was also carried out to confirm and well describe the clusters above reported. As expected, the first two PCs showed four main clusters, grouping the different genotypes in agreement with cluster analysis (Figure S3). Genetic distances among genotypes were confirmed, with five landraces belong to cluster D (Figure 3) showing the highest genetic variability, and the landraces grouped in cluster B and C with a common genetic background. As expected, cuc1 landrace is separated from the others. Unlike cluster analysis, brc-b1 appeared similar to cuc1, between the samples belonging to cluster A and D, without a clear assignment (Figure S3).

The differences among cultivars were further confirmed by fast STRUCTURE analysis. Indeed, this analysis confirmed Cluster and PCoA results, and was able to separate the ancient cultivars form the others. The optimum number of genetic clusters (K) within the collectionwas determined as K=7 (Figure 4). Cuc1 hexaploid landrace belongs to a private group (red pool) and other two pools,

green and gold, overlapped to the clusters B defined in the UPGMA analysis (clustering). Likewise, pool blue of STRUCTURE analysis overlapped to cluster D. By contrast, cluster A in the UPGMA harbored the purple pool except the cultivar Claudio that shared with tri2 a different genetic pool (light blue) in the structure analysis (Figure 4). Thirty out of 32 genotypes (94%) classified into one of the seven pools, using 80% cut-off ancestry. As a rule, rather all varieties showed 100% membership to their group (*K*), except bivc, sco4 and tre2. Among landraces, only brc-b1and bia1 showed a high admixture profile between gold and green, purple and blue pools, respectively (Figure 4).

Figure 4. Admixture proportions of the wheat germplasm collection estimated by



fastSTRUCTURE (K= 7). Each vertical bar represents a sample. The color proportion for each bar represents the posterior probability of assignment of each individual to one of seven groups of genetic similarity. The range of assignment probability varies from 0 to 100%.

3. Conclusion

Here, we investigated a durum wheat landraces collection effectively conserved and available at the "Experimental Sicilian Station for Durum Wheat" (*ESS*) in Caltagirone (Catania, Italy), the largest treasure of wheat genetic biodiversity including the main genotypes (landraces/populations) derived from Nazareno Strampelli research activity well described by De Cillis [6]. Plant height, general lateness and low harvest index of these old landraces have limited their cultivation to a few marginal areas. The possible reintroduction of old landraces in cereal cultivated areas is highly related to important morphological traits that can be useful for tackling the limited input of marginal areas and organic agricultural systems. Our study is focused to characterize and highlight the genetic potentiality of the Sicilian durum wheat landraces as an important source of variation and for detecting unexplored alleles.

SNP array platforms appeared powerful for evaluating genetic diversity and distinguishing among cultivars/landraces/populations, due to the truthfulness reproducibility of SNP matrices derived. SNP markers can be developed with relatively high-throughput, representing a potent strategy to classify and cluster different landraces in durum wheat. SNP are also able to discriminate among landraces and within populations by isolating small panel of SNP and distinct haplotypes. This SNP

ability might be useful to define efficient strategies for gene banks to avoid the genetic erosion by selection and to organize an efficient system for genetic traceability of wheat end-products to elude commercial frauds and sustain the economic profits for the farmers.

In light of results reported here, the interesting panel of investigated genotypes could represent a platform for parental selection in order to develop high-yield durum wheat lines in breeding programs and for association mapping studies.

4. Materials and Methods

4.1 Plant material and experimental conditions

Twenty-seven durum and one bread wheat Sicilian landraces from the collection of the "Experimental Sicilian Station for Durum Wheat" (*ESS*) of Caltagirone (Catania), two historical cultivars (Cappelli and Trinakria) parents of many Italian breeding programs and two modern cultivars (Claudio and Simeto), widespread in Sicilian cereal growing areas were included in this study (Table 4).

Field trials were carried out in 2013 and 2014 in Sicily (Caltagirone, Catania province, 37° 05' 58" N., 14° 29' 56" E., 280 m a.s.l.) in a medium-sandy soil to assess the main bio-productive traits and quality of the grain and wholegrain flours. Genotypes were laid out in the field in 10 mq plots according to a randomized blocks experimental design with three replicates, adopting the typical wheat agronomic management.

ID	Accession	Origin
bb2	Bufala bianca	In collection at the ESS* and sampled in '99-'04 on farm in Randazzo (Catania)
bd3	Bidì	IPK TRI 26213, in collection at the ESS* since 2004 (selection from Tunisian landrace Bidì, line AP4)
bia1	Biancuccia	In collection at the ESS* and sampled in '99-'04 on farm in Salemi (Trapani)
bivc	Casedda Bivona	In collection at the ESS* and sampled in '99-'04 on farm in S. Stefano Quisqinia (Agrigento)
bnc2	Bufala nera corta	IPK3517, in collection at the ESS* since 2004
brc- b1	Bufala rossa corta b	In collection at the ESS* and sampled in '99-'04 on farm in Randazzo (Catania)
cap p1	Cappelli	Old collection of ESS* (selection from Tunisian landraceJenahRhetifah)

Table 4. List of wheat samples characterized by 90K SNP array,	agro-morphological,	phenological
and qualitative traits.		

cas1 pu	Castiglione pubescente	CGN 8213, in collection at the ESS* since 2004	
cic1	Ciciredda	In collection at the ESS* and sampled in '99-'04 on farm in Maletto (Catania)	
cot1	Cotrone	USDA 157975, in collection at the ESS* since 2004	
cuc1	Cuccitta	In collection at the ESS* and sampled in '99-'04 on farm in Fiumedinisi (Messina)	
fsa1	Francesa	Old collection of ESS*	
gig1	Gigante	CGN 8206, in collection at the ESS* since 2004	
gio1	Gioia	IPK 3851, in collection at the ESS* since 2004	
giu1	Giustalisa	USDA 278354, in collection at the ESS* since 2004	
ing2	Inglesa	IPK 3519, in collection at the ESS* since 2004	
mar 2	Margherito	In collection at the ESS* and sampled in '99-'04 on farm in Chiaramonte Gulfi (Ragusa) (selection from Tunisian landrace Mahamuodi)	
mla 1	Martinella	USDA 157971, in collection at the ESS* since 2004	
pao 2	Paola	In collection at the ESS* and sampled in '99-'04 on farm in Randazzo (Catania)	
rea4	Realforte	IPK TRI 28452, in collection at the ESS* since 2004	
reg1	Regina	Old collection of ESS*	
rus1	Russello	In collection at the ESS* and sampled in '99-'04 on farm in Randazzo (Catania)	
sam 3	Sammartinara	USDA 157958, in collection at the ESS* since 2004	
sco4	Scorsonera	In collection at the ESS* and sampled in '99-'04 on farm in Santa Croce di Camerina (Ragusa)	
tim1	Timilia	In collection at the ESS* and sampled in '99-'04 on farm in Maletto (Catania)	
tre2	Trentino	USDA 157965, in collection at the ESS* since 2004	
tri2	Tripolino	Old collection of ESS*	
urr1	Urria	In collection at the ESS* and sampled in '99-'04 on farm in Santa Croce di Camerina (Ragusa)	
val2 gl	Vallelunga glabra	USDA 157979, in collection at the ESS since 2004	
trin	Trinakria	Old collection of ESS*(B.14 x Capeiti 8)	
sim	Simeto	Modernvariety (Capeiti 8 × Valnova)	
cla	Claudio	Modern variety (Cimmyt selection × Durango) × (IS1938 × Grazia)	

* Experimental Sicilian Station for Durum Wheat (Caltagirone, Catania)

4.2 Morphological traits characterization

In accordance to Community Plant Variety Office descriptors [13], twenty-seven morphological and physiological traits were evaluated for each landrace and reference cultivars. Data were collected on ten plants, during vegetative growth (10-60 emergency-earning Zadoks code) and after harvest, on a random sample of fifteen representative spikes (five in each replicate) randomly sampled from each landrace/cultivar.

4.3 Grain and wholemeal flour qualitative traits

Samples of durum wheat grains per each plot were analyzed for the main quality traits. Protein content (% dry matter) was determined by means of Infratec 1241 Grain Analyzer (Foss Tecator, Höganas, Sweden) by near infrared transmittance (NIT) using a calibration based on the Kjeldahl nitrogen method (UNI EN ISO 20483). Calibration was validated in accordance with UNI EN ISO 12099 [56] using different sets of test samples of durum wheat grain with a linear correlation coefficient of r=0.99.

Further, representative samples of grains per each plot were used to measure thousand kernels weight (TKW), test weight (TW) and starchy kernels. TKW was calculated by 15 g of grain weighting and seeds counting by count seeds and comparing them to 1000 seeds. TW was determined with a Test Weight Module (TWM) installed under the Infratec 1241 Grain Analyzer (Foss Tecator, Höganas, Sweden). The most relevant grain defect (percentage of starchy kernels) was finally evaluated, by means of visual estimation on a 30 g grain sample (ICC standard method No. 129) [57].

Grain obtained from each treatment was milled to obtain wholemeal flour by an experimental mill, Cyclotec type 120 (Falling Number, Huddinge, Sweden), with a sieve of 0.5 mm. Physical and chemical gluten features were analyzed by using a Glutomatic 2200, a Centrifuge 2015 and a Glutork 2020 (Perten Instruments AB, Huddinge, Sweden). Wet and dry gluten content and quality (gluten index) were calculated according to the ICC Standard No. 158 [58]. Water binding capacity (WBC) was calculated as difference between the weight of total wet and dry gluten, which gives the water bound in the wet gluten, according to AACCI 38-12.02 [58]. Colorimetric measurements on wholemeal flours were performed following the method described by Sgrulletta et al. [59], which were expressed using the standard CIE 1976 L*a*b* system [60]. Accordingly, the measured indexes were: L* (lightness in the range between black=0 and white=100), a* (the difference between red and green tones) and b* (direct measurement of the yellow color). Those indexes were obtained by means of a CR200 Minolta Colorimeter Chroma (Minolta, Osaka, Japan), using illuminant D₆₅ and a* and b* correspond directly to red and yellow indexes, respectively, while brown index was obtained as 100-L*. Protein, wet and dry gluten content, WBC and starchy kernels were analysed using arc sine transformation [61] prior to analysis of variance.

4.4 Statistical analysis for morphological and qualitative traits

The Hierarchical analysis of variance (ANOVA) was calculated for eleven merceological and quality parameters of seed and semolina and a post hoc Tukey' test was adopted to compare means of 32 durum and bread wheat landraces/cultivars using XLSTAT version 2018.3. Percentage data were normalized expressing them as arc sin root square.

Principal Component Analysis (PCA) was performed using the R package FactoMiner [62]. Finally, the Pearson correlation coefficient (p<0.05) was also calculated among qualitative traits using Hmisc R/package (https://cran.r-project.org/web/packages/Hmisc/index.html) and a scatter plot with the correlation coefficients and their significance was developed with Performance Analytic R/package (https://cran.r-project.org/web/packages/PerformanceAnalytics/index.html).

4.5 DNA extraction and SNP genotyping

Genomic DNA was extracted from fresh leaves/young seedlings of each landrace and cultivar. The GenElute Plant Genomic DNA Miniprep Kit (Sigma-Aldrich, St. Louis, MO) was used and DNA quantity and quality were checked by electrophoresis (1% agarose gel) and NanoDrop® ND-1000 (Thermo Scientific), respectively. DNAs were delivered to TraitGenetics GmbH (Gatersleben, Salzlandkreis, Germany) for genotyping. Two hundred nanograms of genomic DNA were used as template for each reaction, following the manufacturer's instructions (Illumina Inc.). SNP data matrix was generated from the custom Illumina by wheat 90k SNP array (Illumina Inc., San Diego, California), which assays 81,587 SNPs [21].

4.6 90K chip array SNP-data analysis

Raw data were visualised and analysed by using GenomeStudio V2011.1 software (Illumina), with the polyploid clustering version 1.0.0. The dataset was filtered and standardized as previously reported [63]. SNPs with MAF > 0.05 and missing rate < 0.20 were used for all subsequent analyses. PLINK [64], R/snpStats [65] and R/HierFstatpackages [66] were used to compute the main genetic parameters. To investigate the genetic relationships among landraces/cultivars, cluster analysis and Principal Coordinates Analysis (PCoA) were carried out by using R/adegenet 1.3 [67]. To highlight the number of putative genetic pools (*K*) available in Sicilian wheat germplasm FastStructure in the admixture model was used [68]. The analysis was performed as described in Mercati et al [69], using the input files (.bed, .bim, .fam) generated by PLINK.

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Authors' contributions: MCF, FM, MM, MEP and FS conceived and participated in the design of the study and discussed the results critically. MCF, FM, MD and AL performed DNA extraction, SNP genotyping and data

analysis. AS performed the quality traits analyses. SB and GV furnished the ancient wheat landraces. SB, GV and GP performed the morphological analyses. MCF drafted the manuscript. MCF, FM, AS, MD, AL, MPE and FS revised critically it. All the authors read and approved the final manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

Availability of data and materials: the SNP profiles are available at the Dryad repository under a unique identifier doi

Abbreviation

SNP: Single nucleotide polymorphism;.

Supplementary Material

Table S1. Morphological descriptors utilized for characterizing the Sicilian wheat germplasm collection (UPVO/CPVO, 2011).

Figure S1. Gluten index in old and modern durum wheat germplasm. Different letters are significantly different at P < 0.01.

Figure S2. A) Person correlation matrix of 11 qualitative traits. Positive correlations are displayed in blue and negative correlations in red color. Color intensity and the circle sizes are proportional to the correlation coefficients. The significant correlations (p<0.05) were highlighted. **B**) Scatter plot matrix with the correlation coefficients between variables and their significance levels. The distribution of each variable is shown on the diagonal; the bivariate scatter plots with a fitted line are displayed on the bottom of the diagonal; the correlation values and the significance level as stars were highlighted on the top of the diagonal. Asterisks indicate significance levels.

Figure S3. Principal Coordinates Analysis (PCoA) developed with the SNP array. Cyan points: 27 landraces belonging to ancient wheat germplasm of Sicily; brown points: 2 historical varieties (Cappelli and Trinakria); red points: 2 modern varieties (Simeto, and Claudio); blue point the outgroup (Cuccitta).

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