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**Valorization of tetraploid wheat conservation varieties supply chain
through nutritional, technological and genetic characterization**

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Abstract

Durum wheat (*Triticum turgidum* ssp. *durum*) is one of the most important cereal crops worldwide, particularly in the Mediterranean region. In recent years, growing interest has emerged around relatives of durum wheat—such as Khorasan wheat (*T. turgidum* ssp. *turanicum*) and Polish wheat (*T. turgidum* ssp. *polonicum*)—as well as landraces and historical cultivars from the early 20th century, commonly referred to as conservation varieties. This renewed attention is largely driven by consumers' perception that these traditional wheats produce foods that are healthier and safer than those made from modern cultivars. Additionally, cultivating these ancient wheats reinforces the cultural heritage of regions like Sicily, and they may possess unique agronomic or nutritional traits valuable for breeding programs.

In these contexts, it is crucial to explore the differences in kernel composition of these genotypes, through comparative biochemical studies. Moreover, given the economic value of the supply chains of these varieties, it is important to develop methods for varietal identification and traceability.

To address these objectives, in this dissertation four different studies were conducted.

1. Comparative Evaluation between durum wheat and its relatives: Various genotypes of durum, Khorasan, and Polish wheat were assessed for kernel accumulation of dietary fiber and minerals, technological quality, and root morphology. The results revealed that observed differences were more genotype-dependent than subspecies-dependent, indicating that broad generalizations across subspecies are not warranted.
2. Gluten Protein Composition in Sicilian Conservation Varieties: The gluten protein profiles of 51 accessions representing 22 Sicilian conservation varieties were analysed to evaluate intra-accession and intra-varietal variability. The electrophoretic patterns of gliadins proved useful in distinguishing between varieties, offering a promising tool for traceability purposes.
3. Multi-Environmental Trial on Five Sicilian Conservation Varieties: Five prominent conservation varieties—Bidì, Russello, Perciasacchi, Timilia reste bianche, and Timilia reste nere—were cultivated across two Sicilian locations

over three years, alongside three modern cultivars (Iride, Orizzonte, and Simeto). The study quantified levels of two compounds associated with adverse reactions to wheat: amylase/trypsin inhibitors (ATI) and fructans. The differences in their accumulation were generally minor and more strongly influenced by genotype.

4. Genetic Characterization and Development of a DNA-Based Traceability Method:
A total of 141 accessions from the five conservation varieties were genotyped using SNP markers. The resulting data were used to analyse genetic diversity and to develop a robust DNA-based method for varietal identification and traceability.

Overall, this dissertation underscores the importance of considering genotypic variation when evaluating traditional versus modern wheats. These findings, along with the traceability methods developed, can contribute significantly to the valorization of conservation varieties and help to fill the knowledge gap on the differences on kernel composition introduced by breeding.

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1 Introduction

1.1 Wheat

Wheat is one of the most cultivated crops in the world and it is the major staple food in the human diet (Shewry, 2009). Among cereals, in 2023 it was the third for production, with 798.98 Mt, and the first for area harvested, with 220.41 Mha, worldwide (Figure 1) (FAOSTAT 2023).

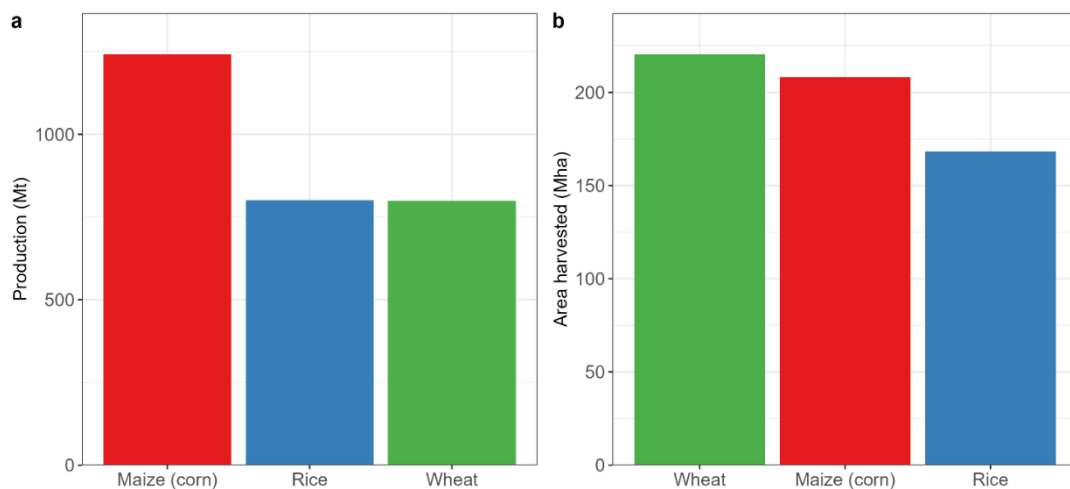


Figure 1 – a) Production (Mt) and b) Area harvested (Mha) of the top three cereals worldwide: wheat, maize and rice (FAOSTAT 2023).

One of the main reasons for its success is its high adaptability to different climatic conditions, making it the crop with the widest range of cultivation area. Moreover, doughs formed from wheat flours have unique properties, which allow them to be processed in a range of baked products, pasta and processed foods (Shewry, 2009).

Wheat is the name used to indicate different species belonging to the *Triticum* genus. Almost 95% the cultivated wheat is hexaploid common wheat (*Triticum aestivum* L.), while the remaining 5% is mainly represented by tetraploid durum wheat (*Triticum turgidum* ssp. *durum* (Desf.) Husn). Common wheat and durum wheat are mainly used, respectively, for bread and pasta production.

Domestication of wheat is a pivotal event in the history of humanity (Salamini et al., 2002). It started about 12000 years ago in the Fertile Crescent and, after a slow speciation process, wheat species started to spread across Eurasia (Zhao et al., 2023).

Today, wheat remains one of the primary sources of calories and protein for humans, contributing approximately 20% to the global intake of both. The wheat kernel is composed in fact mainly of starch (about 60-70%), but it also contains a significant amount of protein (about 10-15%), making it the most important cereal for protein intake (Shiferaw et al., 2013).

1.2 Origin and phylogenesis of wheat

Wheat belongs to the *Triticum* genus (*Triticeae* tribe, *Poaceae* family) and has a complex origin, characterized by different events of hybridization with the *Aegilops* genus followed by allopolyploidization (Figure 2) (Goncharov, 2011).

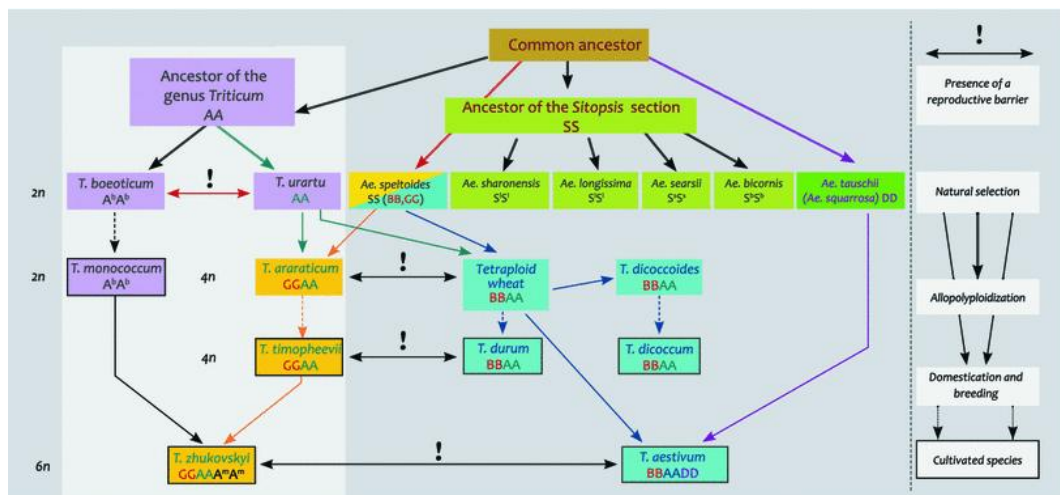


Figure 2 – Phylogenetic scheme of the origin of wheat (Goncharov, 2011).

The *Triticum* genus includes in fact diploid ($2n=2x=14$), tetraploid ($2n=4x=28$) and hexaploid ($2n=6x=42$) species. *Triticum* and *Aegilops* diverged from a common ancestor with seven pairs of chromosomes about 6.5 million years ago (Ma) (Marcussen et al., 2014). About 1 Ma, the two diploid AA genome species *Triticum boeoticum* Boiss. (A^bA^b) and *Triticum urartu* Tumanian ex Gandilyan (A^uA^u) diverged (Matsuoka, 2011). From the former, it derives einkorn (*Triticum monococcum* L., $2n=2x=14$, genome A^mA^m), the only diploid domesticated wheat. The latter, about 0.8 Ma, was involved in two separate allopolyploidization events with some probably extinct species related to *Aegilops speltoides* Tausch. ($2n=2x=14$, genome SS), that led to the creation of tetraploid wild emmer wheat (*Triticum turgidum* ssp. *dicoccoides*, $2n=4x=28$, genome BBAA) and *Triticum araraticum* Jakubz. ($2n=4x=28$, genome GGAA) (Goncharov et al., 2008). Emmer (*Triticum turgidum* ssp. *dicoccum* (Schränk ex Schübl.) Thell, $2n=4x=28$, genome BBAA)

was obtained from domestication of wild emmer and, together with einkorn, started to spread among farmer communities (Dubcovsky & Dvorak, 2007). Durum wheat (*T. turgidum* ssp. *durum*, BBAA) evolved from domesticated emmer about 6500-7500 years ago and slowly replaced it, becoming the prominent tetraploid wheat (Maccaferri et al., 2019). The diffusion of domesticated emmer in the southwest coast of the Caspian Sea resulted in sympatry with *Aegilops tauschii* spp. *strangulata* ($2n=2x=14$, genome DD), leading to the emergence of hexaploid common wheat ($2n=6x=42$, genome BBAADD) about 11700 years ago (Dubcovsky & Dvorak, 2007; Zhao et al., 2023). Another hexaploid wheat, *Triticum zhukovskyi* ($2n=6x=42$, genome GGAAA^mA^m), raised from a hybridization event between *Triticum timopheevii* ($2n=4x=28$, genome GGAA), obtained by domestication of *T. araraticum*, and *T. monococcum* (Badaeva et al., 2022).

Wheat domestication process involved the modification of some major traits. Wild wheats have brittle rachis, that permits the shattering of the seeds upon maturity, and loss of seed dispersal is considered a major hallmark of domestication. This trait is controlled by *Brittle Rachis 1* genes and it has been discovered that loss-of-functions mutations were selected during domestication, that led to a though non-brittle rachis (Avni et al., 2017). A second major trait that was selected was the free-threshing habit, that it is controlled by a major regulatory gene (*Q-factor*) and two genes controlling glume toughness, *Though glumes* (*Tg*) and *soft glumes* (*sog*) (Peleg et al., 2011). Other important traits for domestication were the increment in seed size, the reduction of number of tillers and reduced seed dormancy (Dubcovsky & Dvorak, 2007).

1.2.1 Durum wheat and its relatives

Durum wheat is the second most cultivated wheat after common wheat, with an annual production of 34.3 Mt (average of 2018-2022) (Blanco, 2024). Canada is the world's largest producer, although half of the global production takes place in the Mediterranean region, mostly in Italy and Turkey (Xynias et al., 2020). Durum wheat is mainly used for pasta production, one of the most popular foods worldwide. Other minor end-products are couscous, bulgur and several types of bread.

There are other free-threshing tetraploid wheat subspecies that are less cultivated and less studied, but that are gaining popularity in the latter years: Khorasan wheat (*T. turgidum* ssp. *uranicum*), Polish wheat (*T. turgidum* ssp. *polonicum*), Rivet wheat (*T. turgidum* ssp. *turgidum*) and Persian wheat (*T. turgidum* ssp. *carthlicum*) (Figure 3) (Laidò et al., 2013).

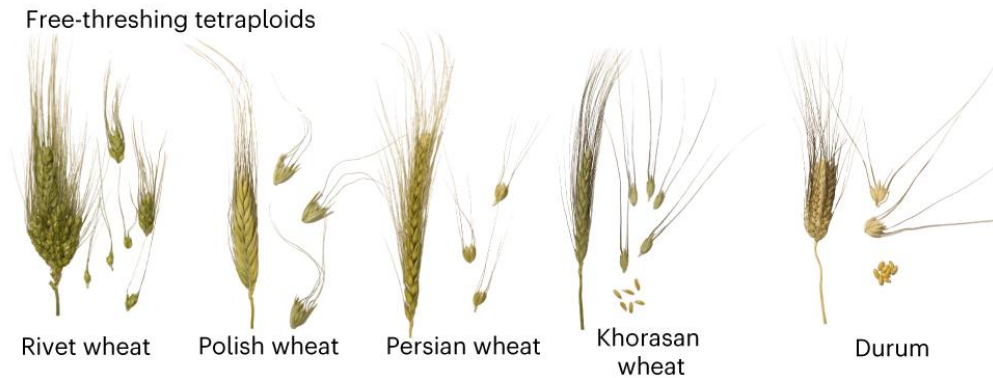


Figure 3 – Major free-threshing tetraploid wheat subspecies (extracted by Zhao et al., 2023).

In particular, Khorasan wheat became popular in the latter years thanks to Kamut® brand wheat, which is a registered trademark of Kamut International Ltd. and Kamut Enterprises of Europe bv, that started to commercialize by this name their variety QK-77 in 1990 (Michalcová et al., 2014). Molecular analyses revealed that Khorasan wheat is genetically related with Polish wheat, maybe deriving from a common ancestor (Laidò et al., 2013; Michalcová et al., 2014). Polish wheat is gaining scientists attention because it accumulates high Zn, Fe and Cu in the seeds and have a high tolerance to Cd and Zn (Y. Wang et al., 2017). Rivet wheat is usually cultivated in mountain environments and it is characterized by cold tolerance (Sicilia et al., 2022). The origin of Persian wheat is controversial, as genetical analyses revealed that it is more similar to common wheat and wild emmer than to durum wheat, suggesting a separate origin, or the presence of gene flow between tetraploid and hexaploid wheat (Laidò et al., 2013).

1.2.2 Genetic diversity of durum wheat

Durum wheat was obtained from human-driven domestication of Emmer wheat about 6500-7500 years ago, but became a prominent crop only 1500-2000 years ago (Maccaferri et al., 2019). Its cultivation was mainly based on landraces until the beginning of 1900, when first breeding programs started in Italy. Initially, they were based on the selection of varieties from landraces originating from North Africa and

South Italy (De Vita et al., 2007). One of the most famous and successful examples of this process was the release of the cultivar Cappelli (syn. Senatore Cappelli) in 1915 by Nazareno Strampelli. It is reported that he selected this variety from the North African landrace Jean Retifah, although recent molecular studies suggest that it was selected from another North African landrace, Bidì (Fiore et al., 2019; Marzario et al., 2018; Taranto et al., 2020). Cappelli played a pivotal role in Italian durum wheat breeding, and it can be considered the main “ancestor” of the modern germplasm (Taranto et al., 2020). Progress in durum wheat genetic improvement were obtained through crossings of the selected cultivars, mostly belonging to *mediterraneum* type, with *syriacum* type landraces, characterized by earliness and semi-dwarfism (Careddu et al., 2023). Finally, the introduction of *Rht* (*Reduced height*) favourable alleles, discovered during the Green Revolution, in the 1980s led to major yield improvements, thanks to resistance to lodging and an increase ability to partitioning biomass to spike (Canevara et al., 1994; De Vita et al., 2007). Moreover, one of the main goals of breeding programs in the last half of the 20th century was also the improvement of technological quality. This was achieved by the selection and the introgression of the most favourable alleles of glutenin subunits (De Santis et al., 2017). However, the numerous improvements achieved through breeding programs have led to erosion of genetic diversity (Mazzucotelli et al., 2020). Genetic diversity was primarily reduced during the initial phases of breeding, when selection processes relied on a limited number of landraces. In more recent phases, however, a slight recovery in diversity has occurred, driven by the introduction of new genotypes into breeding programs (Laidò et al., 2013; Maccaferri et al., 2003; Taranto et al., 2020).

Throughout the years, several approaches have been employed to investigate genetic diversity in durum wheat, such as gluten protein composition analysis, microsatellite (SSR) analysis, and SNP genotyping through microarray technology (Cooke et al., 2003; Janni et al., 2018; Laidò et al., 2013; Maccaferri et al., 2003; Mazzucotelli et al., 2020). The latter has proven to be one of the most efficient and cost-effective methods for studying genetic diversity in wheat, given the complexity of its genome (Maccaferri et al., 2019). Laidò et al., (2013) used both SSR and diversity array technology (DArT) markers to characterize 230 accessions belonging to different *T. turgidum* subspecies and

different years of release. Maccaferri et al., (2019), in parallel with the durum wheat genome sequencing, used the Illumina iSelect 90K SNP array technology on the Global Tetraploid wheat collection, consisting of 1856 accessions of wild and domesticated Emmer wheat and durum wheat landraces and cultivars. Mazzucotelli et al., (2020) used the same array to characterize the Global Durum Wheat Panel (GDP), a collection composed by 2503 accessions of tetraploid wheat originating from diverse geographical regions. Taranto et al., (2020) Illumina iSelect 15K wheat SNP array on 259 accessions of durum wheat cultivated in Italy from 1900 to the present. These are some of the most significant studies on the genetic diversity of durum wheat, and together they shed light on how breeding has shaped its genome over time, highlighting both the erosion and the generation of new genetic diversity.

1.3 Kernel composition

Wheat kernel is composed of three main parts: the endosperm (87–89% of the weight), the embryo (or germ, 2–4% of the weight), and the outer layers (8–10% of the weight) (Figure 4) (Durante et al., 2012).

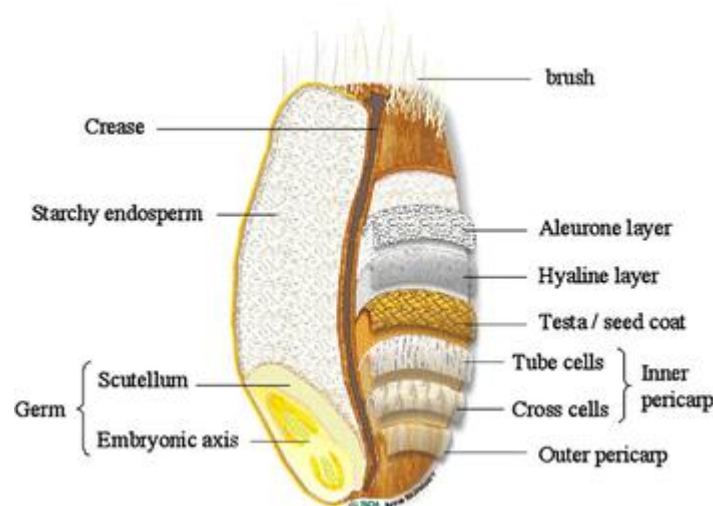


Figure 4 - Histological composition of wheat kernel (Surget & Barron, 2005).

The embryo is a thin-walled structure from which the new plant originates, and it is rich in lipids as well as oxidative and hydrolytic enzymes such as lipases, lipoxygenases, and proteases (Durante et al., 2012). The scutellum separates the embryo from the endosperm and is involved in the mobilization of storage compounds during germination (McKevith, 2004). The endosperm is the main storage tissue and contains starch (60–

70%), storage proteins (8–18%), and smaller amounts of lipids, vitamins, minerals, and non-starch polysaccharides (Shewry, Wan, et al., 2020). The bran includes the pericarp, the testa, and the aleurone layer and is rich in dietary fiber, as well as in minerals, vitamins, and bioactive compounds (Călinoiu & Vodnar, 2018). Traditional milling processes aim to isolate the endosperm, discarding the germ and bran to obtain a more uniform and shelf-stable product (Durante et al., 2012). Wholemeal flours, on the other hand, include all the components of the kernel and are therefore nutritionally richer (Nirmala Prasadi & Joye, 2020). The kernel consists of approximately 70% carbohydrates, of which 54% is starch, 13% proteins, and only 3% lipids (Shewry, 2009).

1.3.1 Carbohydrates

Carbohydrates are the main source of energy for humans, and most of them are consumed through cereals. The wheat kernel contains: 1% monosaccharides (glucose and fructose), 1% oligosaccharides (raffinose and fructooligosaccharides), 1–2% fructans, 65–75% starch, and 10% cell wall polysaccharides (cellulose, β -glucans, and arabinoxylans). The distribution of carbohydrates is not uniform throughout the kernel; in fact, the aleurone and pericarp contain little starch and most of the cell wall polysaccharides, while the endosperm contains about 85% of the starch and only 2–3% of the cell wall polysaccharides (Lafiandra et al., 2014). Cell wall polysaccharides represent most of the dietary fiber of wheat kernel and it is composed of approximately 70% arabinoxylans, 20% β -glucans, 7% glucomannans, and 3% cellulose (Freeman et al., 2017; Marion & Saulnier, 2020). Most of the dietary fiber are concentrated in the bran, which is present only in the less refined flours (Andersson et al., 2013).

1.3.1.1 Starch

Starch is composed of two D-glucose polymers, amylose and amylopectin, which differ in their degree of polymerization and branching frequency. Both polymers consist of D-glucose units linked by α -1,4 glycosidic bonds to form long chains, with branching points formed by α -1,6 glycosidic bonds (Figure 5).

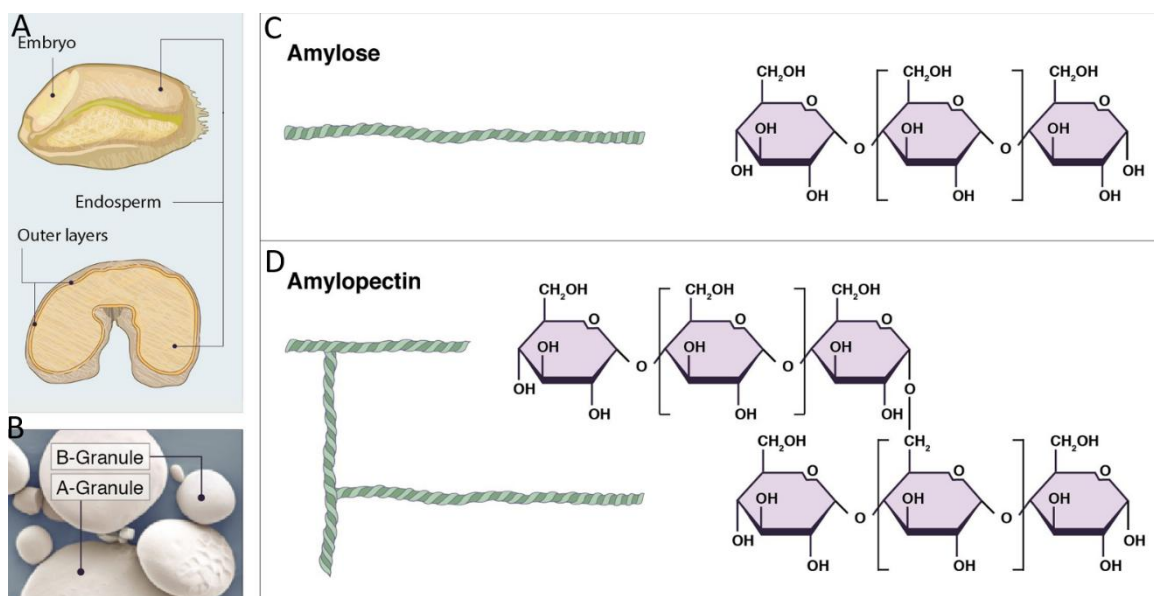


Figure 5 – Representation of: A) wheat kernel; B) starch granules; C) amylose structure and D) amylopectin structure (extracted by Shewry et al., 2020).

Amylose is a linear polymer with a low degree of polymerization (<3000) and a low branching frequency (less than 1%), while amylopectin is a more highly branched polymer with a higher degree of polymerization (>5000) and a branching frequency of 3–4% (Sestili et al., 2014). In wheat, the proportion of amylose in starch ranges from 18% to 35% (Shewry et al., 2020). In plant cells, starch is found in the form of granules enclosed within membrane-bound organelles called amyloplasts. Durum wheat and other wheat species contain starch granules with two distinct structures: type A granules are ellipsoidal in shape and approximately 20–25 μm in diameter, while type B granules are spherical and around 5–6 μm in diameter (Lafiandra et al., 2012). Granules are associated with proteins involved in starch synthesis, known as starch granule proteins (SGPs) (Sestili et al., 2014). The size and distribution of starch granules have a significant impact on technological properties; it has been shown that type B granules have a greater water-holding capacity, which, for example, allows for reduced weight loss during bread baking (Sestili et al., 2014). Moreover, in durum wheat, an increased content of type B granules has been associated with improved pasta texture (Soh et al., 2006).

Starch is of primary importance for humans, contributing to 50–70% of dietary energy intake; however, recent studies have shown that excessive consumption of highly digestible starch is linked to the development of diet-related diseases (Copeland et al., 2009). The rate at which starch is digested depends on its composition; in fact, amylose

is digested more slowly than amylopectin due to its structure being less accessible to the action of human α -amylases (Lafiandra et al., 2014). The amylose-to-amylopectin ratio also affects the technological properties of durum wheat; for instance, pasta produced from lines with reduced amylose content tends to have poorer texture and increased stickiness (Lafiandra et al., 2012).

1.3.1.2 Arabinoxylan

Arabinoxylans (AX) constitute approximately 70% of the wheat cell wall and represent the main source of dietary fiber; their content in the durum wheat kernel ranges from 4.1% to 7.5% of dry weight, although extreme values as low as 2.6% and as high as 12.5% have also been reported (Lafiandra et al., 2012). The majority of AX, approximately 90%, are found in the aleurone layer and pericarp, while only a small amount is present in the endosperm (Marcotuli et al., 2016). Their general structure consists of a linear backbone of D-xylopyranosyl (Xylp) units linked by β -1,4 glycosidic bonds, with side chains of L-arabinofuranosyl (Araf) units attached via α -1,2 or α -1,3 glycosidic bonds (Figure 6) (Wang et al., 2020).

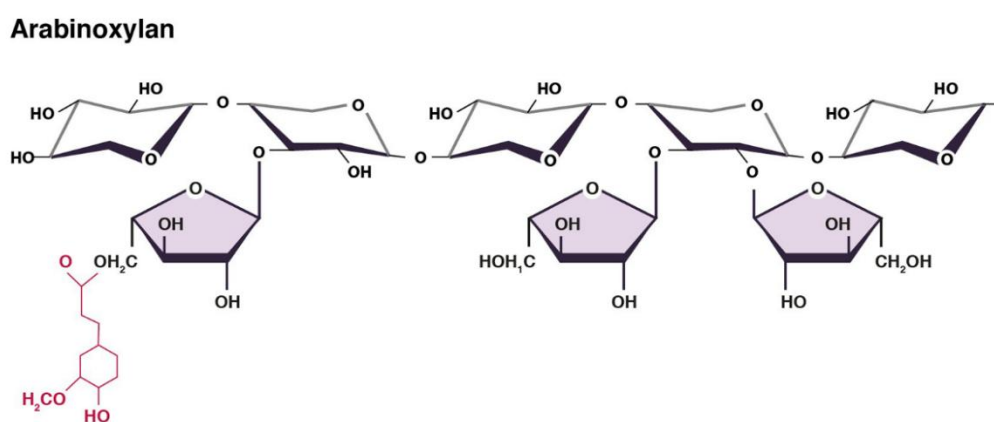


Figure 6 – Arabinoxylan structure in wheat (extracted by Shewry et al., 2020).

A unique feature of AX is the presence of ferulic acid (FA) and p-coumaric acid bound to the 5-OH positions of the Araf terminal residues. FA can form covalent bonds with ferulates from other AX chains, generating diferulate cross-links, or with lignin. The number of cross-links influences the solubility of AX, which depends on the degree of Araf substitution—expressed as the arabinose-to-xylose ratio (A/X)—and their level of feruloylation (Freeman et al., 2017; Shewry & Hey, 2015). Based on their water solubility, AX are classified into water-extractable arabinoxylans (WE-AX) and water-unextractable

arabinoxylans (WU-AX). In cereals, the insoluble fraction is predominant, and in durum wheat it accounts for approximately 80% of the total arabinoxylans (TOT-AX) (Ciccoritti et al., 2011; Marcotuli et al., 2015). The distribution of these two fractions within the grain is not uniform; in fact, the endosperm contains more WE-AX (A/X ratio of about 0.6), while the outer layers contain more WU-AX (A/X ratio of about 1) (Barron et al., 2020). The total AX content in the grain is influenced by both genotype and environment, while the amount of soluble AX depends more strongly on the genotype (Marion & Saulnier, 2020).

1.3.1.3 Beta glucan

β -glucans are glucose polymers linked by β -1,4 and β -1,3 glycosidic bonds (Figure 7).

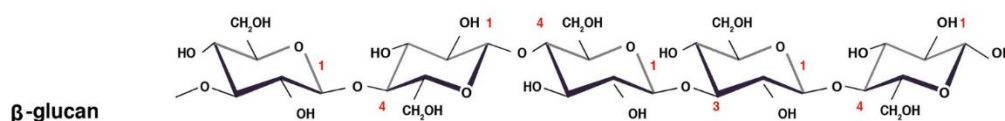


Figure 7 – Beta glucans structure in wheat (extracted by Shewry et al., 2020).

In durum wheat, the ratio between these linkages ranges from 2.5:1 to 3.4:1 (Marcotuli et al., 2020). They account for approximately 20% of the cell wall and are closely associated with cellulose and other wall polysaccharides. Most β -glucans are in the sub-aleurone layer, with only small amounts found in the endosperm (Lafiandra et al., 2012). These molecules are characterized by an irregular and asymmetric structure that prevents intermolecular bonding, making them soluble and highly viscous. Due to their high viscosity, β -glucans are associated with reduced insulin response and lower blood cholesterol levels (Lafiandra et al., 2014). However, wheat β -glucans are present in only small amounts and are also characterized by low solubility; for this reason, their nutritional benefits are more commonly associated with the consumption of oats and barley, which have higher β -glucan content and solubility (Grant et al., 2012).

1.3.1.4 Fructans

Fructans are oligomers and polymers composed of fructose subunits, accounting for approximately 2% of the dry matter in durum wheat (De Gara et al., 2003). Wheat contains a mix of branched fructans of the graminan-type and neotype, both characterized by $\beta(2-1)$ and $\beta(2-6)$ fructosyl linkages (Figure 8) (Cimini et al., 2015).

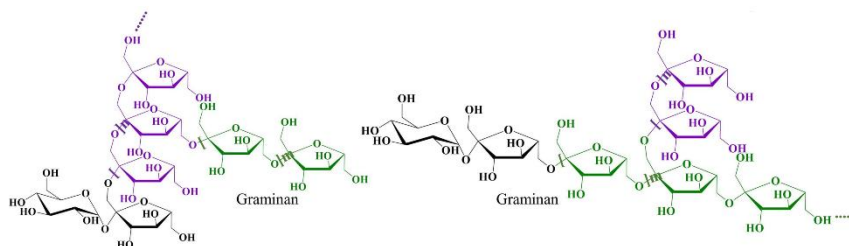


Figure 8 – Graminan- type fructans structure (extracted by Ni et al., 2024). Sucrose units are represented in black, fructosyls units connected by $\beta(2, 1)$ linkage in purple and fructosyls units connected by $\beta(2, 6)$ linkage in green.

Fructans are classified as dietary fiber due to their limited digestibility in humans, and they are known to exert prebiotic effects (Verspreet et al., 2015). However, they may also exacerbate symptoms of irritable bowel syndrome (IBS) and have been implicated in non-coeliac wheat sensitivity (NCWS) (Biesiekierski et al., 2013; Dieterich et al., 2019). Fructans fulfil several functions in plants, including the stabilization of cellular membranes and participation in physiological responses to environmental stress (Cimini et al., 2015). Fructan accumulation in wheat has been reported in drought and cold stress conditions (Bie et al., 2012; Zhang et al., 2015). In fact, fructan are the main water-soluble carbohydrates (WSC) that are accumulated in the stem of wheat and are translocated to the grains during grain filling (Xue et al., 2008). During post-anthesis drought stress photosynthetic processes are inhibited and grain filling depends on accumulated WSC (Sharbatkhari et al., 2016; Yáñez et al., 2017). Fructans promote cold tolerance through the stabilization of the membrane and the prevention of ice crystals formation in the cytoplasm (Hassan et al., 2021).

1.3.2 Protein

Proteins in the wheat kernel serve various roles: i) functional, such as membrane and structural proteins ii) energy storage, such as storage proteins and iii) metabolic, such as enzymes. During seed dormancy, most proteins are immobilized within protein bodies.

During germination, however, the enzymatic machinery is activated, and the proteins are mobilized (Guerrieri & Cavaletto, 2018).

Kernel proteins are classified according to the Osborne system based on their solubility: albumins (soluble in water), globulins (soluble in dilute salt solutions), gliadins (soluble in alcohol), and glutenins (soluble in dilute acids or alkalis) (Lafiandra et al., 2012). Gliadins and glutenins form gluten and are classified as prolamins, a group of cereal proteins characterized by a high content of proline and glutamine (Shewry, 2019). Wheat kernel proteins are also categorized based on their biological function into metabolic and structural proteins (albumins and globulins), and storage proteins (gliadins and glutenins) (Gupta et al., 1996).

Wheat, especially in developing countries, is an important source of protein for humans, despite its relatively low protein content. The nutritional quality of the proteins is determined by their content of essential amino acids, which are amino acids that cannot be synthesized by animals and must therefore be obtained through the diet. Whole wheat grain and flour provides adequate amounts of all essential amino acids recommended for human consumption, except for lysine and methionine, which are present in lower quantities (Shewry, 2009).

1.3.2.1 Glutenin and gliadin

Storage proteins are composed of gliadins and glutenins, and they account for about 80% of the total protein content in wheat. These proteins interact with each other to form gluten, which imparts essential elasticity and extensibility properties to the flour quality (Shewry, 2019). Gluten plays several roles in breadmaking and in pasta-making: in the first case, it must ensure the extensibility and elasticity of the dough to expand and retain the carbon dioxide formed during fermentation and cooking; in the second case, it must be tough enough to hold the gelatinized starch granules during cooking (Sissons, 2008). Gliadins, which account for about 30-40% of the total protein content in wheat, are a heterogeneous group of monomeric proteins, as they only form intramolecular disulfide bridges and are soluble in 70% ethanol (Urade et al., 2018). These are divided based on their electrophoretic mobility at acidic pH (A-PAGE) into: ω -gliadins (which lack cysteine residues and therefore do not form disulfide bonds), γ -gliadins, β - and α -gliadins (the latter two are closely related and are now commonly

referred to collectively as α -type gliadins) (Troccoli et al., 2000). Glutenins, on the other hand, are polymeric proteins whose subunits are linked by intermolecular disulfide bonds (D'Ovidio & Masci, 2004). These polymers are too large to be separated by conventional electrophoresis; however, reduction of the disulfide bridges allows separation of the subunits by SDS-PAGE into: high molecular weight subunits (HMW-GS) and low molecular weight subunits (LMW-GS), which are further divided into types B, C, and D (Shewry, 2019). Another widely used classification divides the gluten proteins based on structural characteristics into: HMW-GS prolamins, which include only the HMW subunits; sulphur-poor prolamins, which include ω -gliadins; and sulphur-rich prolamins, which include α - and γ -gliadins and LMW-GS (Lafiandra et al., 2012). A summary of gluten proteins classification is reported in Figure 9.

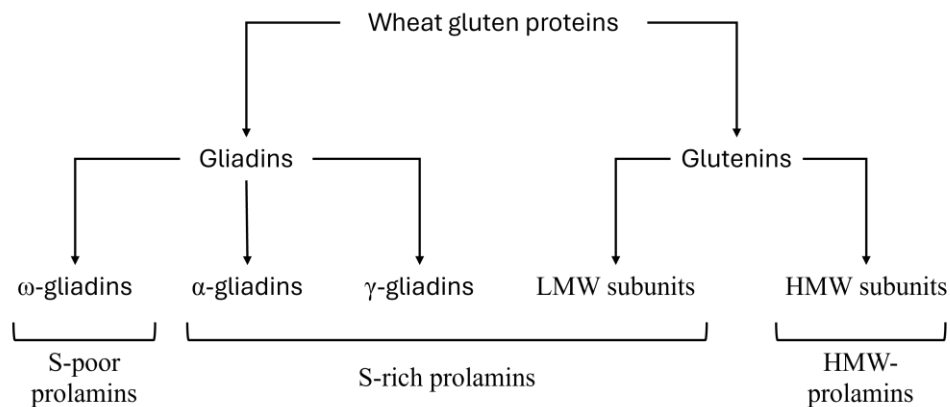


Figure 9 – Gluten proteins classification (Lindsay & Skerritt, 1999).

Genes encoding gluten proteins are present on the chromosomes of group 1 and 6 of wheat (Figure 10).

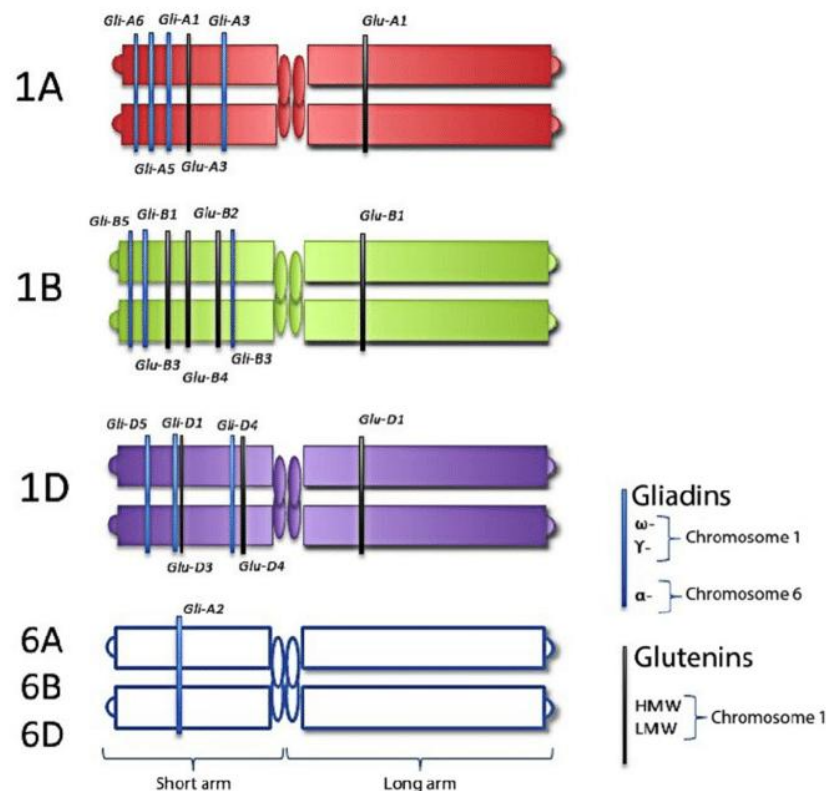


Figure 10 – Location of the genes encoding gluten proteins in common wheat (Giménez et al., 2014).

HMW-GS are encoded by the *Glu-1* loci, each containing two genes encoding x-type and y-type HMW-GS, present in the long arm of the group 1 chromosomes (Shewry et al., 1992). Durum wheat typically expresses two or three subunits, because *Glu-A1y* is always silenced, while *Glu-A1x* can be silenced or not. Bread wheat can express from 3 to 5 subunits (Shewry & Lafiandra, 2022). LMW-GS are encoded by various loci located in the short arms of group 1 chromosomes, linked to gliadin loci. *Glu-3* is the most studied locus and encodes for B-type LMW-GS, the most abundant type (Roncallo et al., 2021). Gliadin genes are located in the short arm of chromosomes groups 1 and 6 and are highly polymorphic (Barak et al., 2015).

The properties of doughs, such as elasticity and extensibility, depend on the allelic forms of the gluten proteins and their quantity; in fact, favourable alleles allow the formation of a strong dough with good leavening and stability characteristics (Ma et al., 2019). The quality of semolina products is influenced both by the quantity and composition of the gluten proteins. A high protein content is important for pasta-making because the

protein matrix plays the role of retaining the starch granules during cooking; this helps prevent the pasta from becoming sticky, ensures it holds its shape during cooking, and prevents it from breaking. The composition of gluten also affects the quality of pasta, and a stronger gluten gives the dough better toughness, preventing the loss of starch granules (Sissons, 2008). The strength of gluten is also important for bread-making, as it enhances the viscoelastic properties of the dough, allowing it to support the formation of carbon dioxide during fermentation (Mefleh et al., 2019).

Several methods are available for the qualitative assessment of durum wheat semolina. The Gluten Index measures the ability of gluten to pass through a sieve under centrifugal force and is directly correlated with gluten strength (ISO, 2015). The Alveograph test evaluates the resistance of semolina dough to extension, and it is based on the insufflation of air in a dough disk until breakage. Three major parameters of the dough are obtained from this test: the strength (W), the tenacity (P) and the extensibility (L). High values of W and of the ratio P/L have a positive correlation with technological quality and are desirable for pasta production (Cecchini et al., 2021).

The limit of these tests is that they require a large amount of sample, often not available in early stages of breeding programs. To overcome this, some predictive methods were developed: the SDS sedimentation test and the calculation of the percentage of unextractable polymeric proteins (%UPP). The first one relies on the ability of gluten protein to swell (forming a sediment) in acidic solutions and it's an approved method by the Cereal & Grains association (AACC approved method 56-70.01). The second one is based on size-exclusion HPLC and allow to calculate the proportion of polymeric proteins not extractable in a solution containing SDS, consisting of large glutenin polymers (Gupta et al., 1993, 1995). The %UPP value is positively correlated with gluten strength and dough viscoelastic properties (Sissons et al., 2005).

1.3.2.2 Alpha-amylase/trypsin inhibitors

Alpha-amylase/trypsin inhibitors (ATI) are water-soluble (non-gluten) proteins accounting for about 4% of the kernel proteins and their role is to protect the kernel from insect and mammalian amylase (Dupont et al., 2011). ATI are encoded by at least 19 genes and can be divided in four groups: monomeric (0.28), dimeric (0.19, 0.53), tetrameric (CM1, CM2, CM3, CM16, CM17) and monomeric trypsin inhibitor (CMX1/3,

CMX2) (Geisslitz et al., 2021). Additional protease inhibitors with sequence similarities to ATI are also present in wheat, such as wheat trypsin inhibitor (WTI), wheat chymotrypsin inhibitor (WCI), and wheat endogenous amylase/subtilisin inhibitor (WASI) (Altenbach et al., 2011). All ATI are predicted to share the same overall structure: 4 α -helices connected by irregular loops, a short antiparallel β -sheet and 4-5 intrachain disulfide bonds (Figure 11).

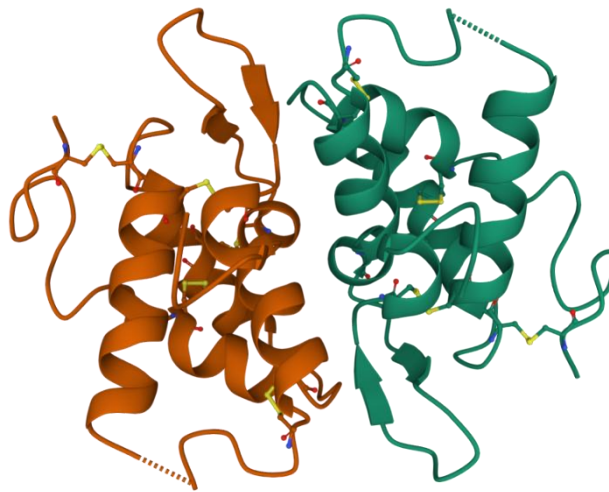


Figure 11 – 3D structure of homodimeric 0.19 ATI (PDB entry 1HSS; Oda et al., 1997). The ball-and-stick indicates the disulfide bonds. Image created using Mol* (Sehna et al., 2021).

ATI are also considered as the major trigger of immune response in wheat allergies, especially bakers' asthma and as putative trigger of autoimmune response in CD (Junker et al., 2012; Sander et al., 2015). ATI are also suspected to play a role in Non-Celiac Wheat Sensitivity (NCWS) (Geisslitz et al., 2021). NCWS is characterized by intestinal (diarrhea, abdominal pain, bloating) and extra-intestinal (dermatitis, fatigue, headaches) symptoms and there is strong evidence that it's caused by the activation of innate immunity in response of wheat proteins (Schuppan et al., 2015). Noteworthy, it has been demonstrated that the ingestion of α -Amylase/trypsin inhibitors (ATI) induces potent innate immune response and the activation of the myeloid cells via the interaction with the TLR4–MD2–CD1 complex (Junker et al., 2012; Zevallos et al., 2017).

1.3.3 Mineral and vitamins

Vitamins in the durum wheat kernel are mainly concentrated in the pericarp, aleurone layer, and germ. These include various B-group vitamins—B1 (thiamine), B2 (riboflavin), B3 (niacin), B6 (pyridoxine)—as well as vitamin E and carotenoids, which are precursors of vitamin A (Grant et al., 2012). B-group vitamins play important roles in various metabolic processes, particularly carbohydrate metabolism (thiamine), and protein and fat metabolism (pyridoxine and riboflavin), and are highly bioavailable in cereals (Batifoulier et al., 2006). Vitamin E is present in the kernel as tocopherols and tocotrienols and is important for human health due to its antioxidant and cholesterol-lowering properties (Durante et al., 2012). Durum wheat is also a good source of minerals: it is low in sodium and high in potassium, and contains appreciable amounts of iron, zinc, magnesium, copper, manganese, molybdenum, and selenium (Grant et al., 2012). Wheat, together with other cereals such as oat and barley, contributes significantly to the intake of iron and zinc; for instance, in the United Kingdom, it provides 44% of the daily iron intake and 25% of the daily zinc intake (Shewry, 2009). Selenium, another trace element essential for human health, is present in durum wheat as selenomethionine, which increases its bioavailability (Grant et al., 2012). It is important to note that both vitamins and minerals are mainly located in the bran and are therefore present only in small amounts in refined flour (Shewry et al., 2012).

1.3.4 Lipids

Durum wheat kernels contain about 3% lipids, of which 66% are found in the embryo, 15% in the aleurone, and approximately 20% are distributed in the endosperm, partly bound to the starch granules. Their composition and content depend on both genetic and environmental factors, and in general, durum wheat has a higher content compared to common wheat (Narducci et al., 2019). Lipids have a significant impact on the nutritional and technological quality of flour, affecting the rheological and oxidative properties of dough; moreover, their volatile compounds contribute to the aroma of processed products (Flagella, 2006). In durum wheat, about 70% of the total lipids are non-polar lipids, most of which are triglycerides, but their composition has not been fully studied yet. The embryo has a high content of unsaturated fatty acids (about 80% of total fats), mainly composed of linoleic acid (18:2) and linolenic acid (18:3), both essential for

the human body since it cannot synthesize them. Both are precursors of prostaglandins, hormones that play an important role in muscle contraction and inflammation processes (Lafiandra et al., 2012). Linoleic acid also promotes cholesterol excretion and is a precursor of phospholipids.

Wheat is also a source of phytosterols, which contribute to lowering the concentration of total lipoproteins; several studies have shown that durum wheat contains a higher percentage of phytosterols compared to common wheat (Grant et al., 2012). Non-polar lipids have a negative impact on the final bread volume, destabilizing the film formed by soluble proteins that is responsible for retaining gases. Polar lipids, on the other hand, have a positive role in leavening as they stabilize the interface with the gaseous phase by binding to the albumin fraction. Monoacyl lipids bind to starch granules, affecting their thermal properties, improving the crumb texture of the bread, and enhancing its stability during storage (Marion & Saulnier, 2020). The lipid fraction of the grain also contains pigments responsible for the colour of semolina and pasta, which are divided into carotenoids and flavones. Durum wheats have a higher content of yellow pigments compared to hexaploid wheats, and this is an essential characteristic for determining certain qualitative aspects of pasta (Flagella, 2006). The intensity of yellow in the kernel and its products is influenced both by carotenoid biosynthesis and their degradation during processing, which is caused by oxidative enzymes (Ficco et al., 2014).

1.4 Ancient grains, landraces and old varieties

In recent years, there has been growing consumer interest in the so-called 'ancient grains' (Wendin et al., 2020). This term is generally used to refer both to wheat-related species such as einkorn, emmer, spelt, and khorasan, as well as to landraces and traditional varieties of durum and bread wheat that were cultivated before the Green Revolution. This definition is overly broad, as it groups together species that are often hulled with varieties that have been grown over the past century. It is therefore more appropriate to refer to the latter as 'heritage' or 'heirloom' wheats (Brouns et al., 2022).

The consumers interest in ancient grains is mainly given by the perception that they are healthier than their modern counterpart. Unfortunately, most of the beliefs on ancient grain are not scientifically proven. For example, contrary to the widespread belief that

breeding has increased the toxicity of wheat gluten, numerous studies have shown that the levels of gluten, gliadin, and CD-active epitopes have remained stable over time. In some cases, "ancient grains" even exhibit higher concentrations of these components compared to modern cultivars (Brouns et al., 2022).

There are studies suggesting that the consumption of ancient grains may be safer, especially for individuals suffering from IBS and NCWS (Alvisi et al., 2017; Dinu et al., 2018; Seidita et al., 2022; Sofi et al., 2014; Spisni et al., 2019; Valerii et al., 2015; Whittaker et al., 2015, 2017). However, as exhaustively reported by Brouns et al. (2022) and Shewry (2018), these studies lack of adequately comparison with modern varieties and the effects of agronomical practices and environmental conditions on bioactive compound accumulation are not taken into consideration.

In this context, comparative studies on grain composition between ancient and modern wheat are essential to evaluate these assumptions and to explore potential physiological differences associated with their consumption. Tendentially, protein and gluten contents are higher in ancient grains (einkorn, emmer and spelt) than in common wheat (Geisslitz et al., 2019). Analysing old (year of registration between 1900 and 1960) and modern wheat varieties, Shewry et al., (2020) found that protein content is lower in the latter in favour of starch content. In general, breeding decreased gliadin content in favour of glutenin content, not altering the content of CD-active epitopes (Pronin et al., 2020, 2021). Common wheat, spelt and emmer have an ATI content higher than durum wheat, while einkorn ATI content is extremely low, nearly undetectable (Geisslitz et al., 2020). Three different studies on common wheat landraces and modern varieties demonstrated that breeding did not alter total ATI content (EL Hassouni et al., 2021; Geisslitz et al., 2023; Jahn et al., 2025). Einkorn and common wheat have a fructan content higher than emmer and durum wheat (Ziegler et al., 2016). Breeding did not even alter fiber content in common and durum wheat (De Santis et al., 2018; Lovegrove et al., 2020). Lovegrove et al. (2023) showed that emmer and spelt have a higher content of protein, iron, zinc, magnesium, choline and glycine betaine, asparagine and raffinose than common wheat, but lower content of arabinoxylan and beta glucans.

As expected, greater differences are observed between modern wheat and relative species, such as einkorn emmer and spelt, than between modern cultivars and landraces

or historical varieties. Comparative studies on grain composition of these varieties are fundamental to make any statement about their nutritional and healthy properties.

1.5 Tetraploid wheat conservation varieties in Italy

Italy is one of the main producers of durum wheat and the first producer of pasta worldwide (Blanco, 2024). This crop is especially important for this country and their histories are tightly connected. Italy has played a foundational role in the development of durum wheat cultivation, with the earliest formal breeding programs dating back to the early 20th century. At that time, farmers primarily relied on landraces that had adapted over generations to the specific climatic and agronomic conditions of southern Italy, especially Sicily. These initial breeding efforts focused on selecting pure lines from both local landraces and those introduced from North Africa. One of the most well-known outcomes was the release of the Cappelli variety (also known as Senatore Cappelli) in 1915 by Nazareno Strampelli, selected from the Tunisian landrace Jean Retifah. In the decades that followed, breeding programs led to the release of several new varieties, primarily of the *mediterraneum* type. Around the mid-20th century, breeders began crossing these with *syriacum* type landraces, resulting in varieties with earlier flowering and semi-dwarf growth habits (Giunta et al., 2007). The Green Revolution further transformed durum wheat production by introducing reduced height (*Rht*) genes, which significantly boosted yield potential (De Vita et al., 2007). Despite these advancements, traditional landraces and older cultivars gradually fell out of commercial use and survived only through the efforts of dedicated farmers and research institutions—often without formal conservation measures, exposing them to the risk of genetic erosion. In recent years, however, interest in these ancient varieties has resurged, driven by consumer demand for traditional and locally adapted crops, as well as scientific recognition of their genetic value. While breeding has greatly improved productivity, it has also led to a narrowing of genetic diversity (Mazzucotelli et al., 2020; Taranto et al., 2020). To address this, the Italian government adopted specific legislation aligned with EU Directive 2008/62/EC, first through Legislative Decree No. 149 of 2009 (later replaced by Decree No. 20 of 2021), which made it possible to register "conservation varieties"—defined as traditional landraces or varieties adapted to specific local conditions and at risk of disappearing—within the National Catalogue of

Agricultural Species. As of today, 28 durum wheat conservation varieties have been registered, 24 of which are Sicilian. Sicily continues to be a key player in durum wheat cultivation, ranking as Italy's second-largest producing region. Here, many historical varieties are still cultivated in low-input systems, particularly in marginal lands where their resilience and stable yields are especially valued. Products made from these grains are often positioned in premium markets due to their quality and traditional appeal (De Vita et al., 2010; Varia et al., 2021).

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2 Aim of the work

The aim of the work is the characterization of durum wheat relatives, landraces and historical varieties for their valorisation and conservation. Their cultivation is not widespread because of their poor yield and technological quality in comparison to modern durum wheat varieties. However, lately, they gained an increasing attention because they often can be cultivated in marginal areas and in low-input agricultural systems. Their genetic diversity could be useful in breeding programs, because they often harbour genes useful for abiotic and biotic stresses tolerances. These varieties are also being rediscovered by consumers, both because they appreciate their sensory quality and traditional value, and because there is a common belief that they are healthier and safer than modern counterparts. At the moment, there are no scientific proofs that validate this theory. Moreover, there is a lack of comparative studies that explore compositional and genetic differences between these old genotypes and the modern cultivars. The increasing value of the supply chain of these crops also highlights the need to develop methodologies for their proper identification and conservation, as well as to ensure their traceability. This dissertation is composed of four different studies, divided in chapters, with the aim to shed a light on all these aspects.

In Chapter I, we performed a comparative study between five accession of Khorasan wheat (*T. turgidum* ssp. *turanicum*), five accessions of Polish wheat (*T. turgidum* ssp. *polonicum*), two modern and one old durum wheat cultivars. Khorasan wheat is one of the most popular relatives of durum wheat, mostly because of the commercial success of Kamut®, and it is characterized by the higher length of the kernels. Polish wheat is less popular, but it is reported to be able to accumulate more microelements in comparison with durum wheat. The aim was to verify if these durum wheat relatives are nutritionally better, in particular we analysed their dietary fiber and mineral content, and have a comparable technological quality. Moreover, we evaluated their root morphology and their ability to uptake iron. To evaluate environmental effects, the varieties were cultivated for two years in Central Italy (experimental educational farm “Nello Lupori” of University of Tuscia, Viterbo).

Chapter II, III and IV shifted the focus on Sicilian tetraploid conservation varieties. Sicily is an Italian region particularly rich in tetraploid wheat landraces and it was fundamental for the first durum wheat breeding programs at the beginning of the 20th century. 24 of the 28 tetraploid wheat conservation varieties registered in the Italian national catalogue for agricultural species are Sicilian and they are still cultivated, boosting the local economy.

In chapter II, fifty-one accessions belonging to twenty-two Sicilian conservation varieties were collected from the Council for Agriculture Research and Economics, Plant Protection and Certification Centre (CREA-DC) collection and grown for two years in Sicily. Their gliadin and glutenin subunits electrophoretic pattern were analysed to measure intra- and inter- varietal variability and to verify its possible employment for traceability. Moreover, their percentage of unextractable polymeric proteins (%UPP) was calculated for technological quality prediction.

In chapter III, the five most important Sicilian conservation varieties (Bidì, Perciasacchi, Russello, Timilia reste bianche, Timilia reste nere) were selected. They were cultivated, together with three modern varieties (Iride, Orizzonte, Simeto) in two locations in Sicily for three years and their amylase/trypsin-inhibitors (ATIs) and fructan content was quantified. ATIs are the cause of some wheat allergy and are putatively involved in non-celiac wheat sensitivity (NCWS). Fructans are also putatively involved in NCWS, and they also worsen the symptoms of irritable bowel syndrome (IBS) patients. Considering that old genotypes are considered safer for human consumptions than modern cultivars, often without scientific proofs, our aim was to evaluate if there were differences in the accumulation of these potential unhealthy compounds. As in chapter II, the %UPP of these varieties was also calculated for technological quality prediction.

Finally, in chapter IV a total of 181 accessions of tetraploid wheat, 141 of which are Sicilian conservation varieties, were genetically characterized using a DNA microarray that included 6707 SNPs. The obtained genetic information was used for the diversity analysis of the population and to develop a DNA-based traceability method of the Sicilian conservation varieties Bidì, Perciasacchi, Ruscia, Russello, Timilia reste bianche and Timilia reste nere.

3 Chapter I - Characterization of *Triticum turgidum* spp. *durum*, *turanicum*, and *polonicum* grown in Central Italy in relation to technological and nutritional aspects

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Characterization of *Triticum turgidum* spp. *durum*, *turanicum*, and *polonicum* grown in Central Italy in relation to technological and nutritional aspects

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Introduction: Wheat is a staple food, with the two most common species being *Triticum aestivum* and *Triticum turgidum* ssp. *durum*. Moreover, the latter, *T. turgidum*, includes other tetraploid subspecies, among which the spp. *turanicum* (Khorasan wheat) and *polonicum* (Polish wheat), whose importance has increased in the last decades, representing alternative crops for marginal areas, in addition to being a source of genetic diversity.

Methods: In this work, different accessions of these three subspecies of *T. turgidum* have been grown in 2 years in the same environment and have been characterized for technological properties and factors affecting nutritional quality, such as fiber amount and the content of micro- and macro-nutrients in grains, and for root morphological traits.

Results: These analyses allowed the identification, in particular, of a Polish wheat accession showing better technological performances, a higher amount of positive micro- and macro-elements, and a lower amount of toxic cadmium. The modern variety Svevo and the Polish Pol2 showed the lowest and the highest shoot:root ratio, respectively. The high shoot:root ratio in Pol2 was mainly attributable to the decrease in root growth. Although Pol2 had a lower root biomass, its particular root morphology made it more efficient for nutrient uptake, as evident from the greater accumulation of micro- and macro-nutrients.

Discussion: These results underline that it is not possible to draw general conclusions about the difference between primitive and modern wheats, but rather a case-by-case approach should be chosen.

KEYWORDS

Triticum turgidum, *Triticum durum*, *Triticum turanicum*, *Triticum polonicum*, technological quality, micronutrients, fiber, root traits

1 Introduction

Wheat is one of the oldest cultivated plants and one of the most important sources of food. Under this name, different species are included, two of which are the most commonly grown: *Triticum aestivum*, also known as bread wheat, because mostly used for bread and leavened products, and *Triticum turgidum* ssp. *durum* (also known as *T. durum* or durum wheat), mostly used for pasta or typical Mediterranean foods such as bulghur, couscous, and, in some cases, flat and leavened bread as well.

The genome composition of *T. aestivum* is AABBDD, namely, hexaploidy, whereas that of the tetraploid *T. durum* is AABB. Moreover, *T. durum*, other subspecies of *T. turgidum*, share the same genomic composition, among which there are *T. turgidum* ssp. *polonicum* and *T. turgidum* ssp. *turanicum*, also known, respectively, as *T. polonicum* (or Polish wheat) and *T. turanicum* (or Khorasan wheat, from the Iranian Region where it was first described). They are free-threshing with tough rachis that are easy-harvest wheats, because they have soft glumes and non-hulled seeds (Yang et al., 2022). *T. turanicum* and *T. polonicum* are closely related, and it has been suggested that the first originated from the hybridization with *T. dicoccon* (Michalcová et al., 2014).

The importance of the two subspecies has increased in the last decades, as a consequence of the introduction in the market of KAMUT® brand wheat, an ancient variety of grain (*Triticum turgidum* ssp. *turanicum*) that has reached a high popularity because of its suggested positive effects on human health (Bordoni et al., 2017; Spisni et al., 2020). In the wake of KAMUT® brand wheat, any Khorasan wheat is acquiring importance because it is considered healthy (Geisslitz and Scherf, 2021), with some accessions sold under registered names and others simply described as such, sold as semolina or processed foods, with a wide market, especially in Western Countries where they can be found even in the large-scale distribution.

Furthermore, both Polish and Khorasan wheats, being able to be grown organically and adaptable to different environments, represent attractive and alternative crops for the marginal areas of Mediterranean basin, which were no longer widely cultivated due to the low grain yields compared with modern wheat cultivars. However, traditional (mostly local) farmers have continued to grow them to use in low-input agriculture, and they are a source of genetic diversity (Ganugi et al., 2021). Moreover, they are important for economic reasons, because heritage wheats respond to the demand of a specific niche of consumers, careful to the need of conserving cultural habits, often associated by people with the concept of a healthier lifestyle.

Heritage wheats are typically consumed as whole wheats, and this contributes to their healthiness. In general, they have poor technological properties. This is due to the poor-quality gluten proteins and to the presence of fibers, mostly present in the outer pericarp. Because heritage wheats are mostly consumed as whole-wheat leavened products, this affects gluten formation and gas-retention capacity, because they absorb most of the water available (Li et al., 2014; Cappelli et al., 2019; Cappelli and Cini, 2021).

Regarding health-related compounds, the situation is rather controversial and strongly dependent on the specific genotype taken into consideration (Shewry and Hey, 2015; Prandi et al., 2017;

Ozuna and Barro, 2018; Lovegrove et al., 2020; Shewry et al., 2020), rather than to their belonging to a specific historical period. There is a strong need for further research on this matter, along with a standardization of evaluation procedures, in order to make reliable comparisons (Shewry, 2018).

Khorasan wheat is by far the most known, but Polish wheat has similar characteristics. It is highly adaptable, especially to Mediterranean climates, and, despite the name, it is mostly grown in Andalusia (Rodríguez-Quijano et al., 2003), Italy, Western Ukraine, Southern Asia, Algeria, and Ethiopia (Eticha et al., 2006). It is characterized by a high content of microelements (Bieńkowska et al., 2019) and by a higher amylose content (Rodríguez-Quijano et al., 2003) that accounts for a higher amount of resistant starch that increases the nutritional quality of derived foods.

The aim of this work is the characterization of accessions of Khorasan and Polish wheats, in comparison to durum wheats, grown for 2 years in the same environment, in terms of technological properties and factors affecting nutritional quality, such as fiber amount and the content of micro- and macro-nutrients in grains. This latter aspect will be also related to the root morphological traits of the different genotypes.

2 Materials and methods

2.1 Plant materials

A previous analysis of 50 accessions of Khorasan and Polish wheats (Sestili et al., 2018) allowed to select five accessions of *T. turanicum* and five accessions of *T. polonicum*, which showed higher quality characteristics. They are listed in Table 1, along with the durum wheat cultivars used as references.

Selected materials were grown in 2 years (2018–2019 and 2019–2020) at the experimental educational farm “Nello Lupori” at the University of Tuscia, in three randomized blocks (1.5 m × 2 m), and pools from each block correspond to the biological replicates. Not all the accessions were used for all the analyses, and they are specified in the text.

Whole flour for the gluten protein and nutritional related analyses was obtained by using a Cyclotec mill (Foss Tecator AB, Hoganas, Sweden) equipped with a 0.5-mm sieve, whereas, in the case of technological analyses, a traditional roller milling process (Bühler, model MLU 202, Uzwil, Switzerland) was used. Each grain sample was conditioned by adding water until the moisture value of 17% and left to rest for 24 h. The main milling products were collected: semolina refined through a sieving treatment (sieve types: 38GG, 40GG, and 44GG) using a suitable pilot plant sieving system (NAMAD Impianti, Rome, Italy), bran fractions (coarse and refined types), and fine middling.

2.2 Technological analyses

Grain samples were evaluated for test weight, moisture and protein content, gluten percentage, and yellow color through Near-Infrared analysis in Transmission mode by using Infratec™ mod

TABLE 1 Species, cultivars and accessions used in this paper.

Species	Original accession code or cultivar	New accession code
<i>T. turanicum</i>	PI624217	Tur5
<i>T. turanicum</i>	PI184543	Tur21
<i>T. turanicum</i>	PI576854	Tur26
<i>T. turanicum</i>	QK-77	Khor1*
<i>T. turanicum</i>	Etrusco	Etrusco
<i>T. polonicum</i>	PI191810	Pol2
<i>T. polonicum</i>	PI191808	Pol6
<i>T. polonicum</i>	CLTR5023	Pol11
<i>T. polonicum</i>	AS304	Pol304
<i>T. polonicum</i>	IC12196	Pol2156
<i>T. durum</i>	Aureo	–
<i>T. durum</i>	Svevo	–
<i>T. durum</i>	Senatore Cappelli	–

*Khor1 corresponds to kernels obtained by local field cultivation of KAMUT® brand wheat, purchased by Molino Bongiovanni s.r.l. (Villanova Mondovì, CN, Italy). KAMUT® trademark is used to market and sell the QK-77 variety with certain quality guarantees. Because KAMUT® is a registered trademark of Kamut International Ltd. and Kamut Enterprises of Europe bv, this name could not be used for locally produced kernels because they were obtained out of the official KAMUT® food chain.

1241 (FOSS, Hillerød, Denmark), by using less than 60 s for 10 subsamples, including specific gravity analysis and maximum of 40 s for subsamples, with dynamic subsampling technology.

The evaluation of the yellow color was performed with the reflection colorimeter Konica Minolta Chroma Meter CR-400 with an attachment CR-A50 for the measurements of granular material (space L *, a *, and b *); the color was expressed by yellow index. The average was calculated on the 10 different subsamples. Gluten quantity and quality were evaluated by Gluten Index direct method using Glutomatic System (Perten, Sweden) (ICC 158). The rheological parameters were evaluated by using the Chopin alveograph (model NG) AACC Method 54-30.02 (1999) by performing four replicates. For pasta-making process, a pilot press plant (NAMAD Impianti, Rome, Italy) was used, and the “spaghetti” format (Ø =1.6 mm) has been adopted. The end-products (pasta samples) were submitted to a drying cycle for 18 h at low temperature (50°C) using a suitable dryer plan (AFREM, Lyon, France). Pasta evaluation was carried out using the method modified by D'Egidio et al. (1993). Statistical analyses [analysis of variance (ANOVA) and Tukey test] were performed by using the software PAST (v. 2.12, Oslo, Norway) (Hammer et al., 2001).

2.3 Mineral content

The amounts of zinc (Zn), magnesium (Mg), iron (Fe), copper (Cu), cadmium (Cd), and selenium (Se) were determined by the acid digestion method followed by detection with Inductively Coupled Plasma Optical Emission 184 Spectrometer (ICP-OES). In detail, for each sample, three technical replicates of whole flour were subjected to a first phase of acid digestion using a high-pressure laboratory microwave oven (Mars plus CEM, Italy) with a 1,800-W energy output. A pre-digestion was carried out on 200 mg of dry sample in three consecutive steps: 1 h with 7.5 mL of HNO₃ at 67% v/v, 1 h with 0.5 mL of HCl; 30 min with 2 mL of H₂O₂ to reach a final volume of 10 mL. The acid solution obtained was mineralized within the microwave, increasing the temperature from 25°C to 180°C in 37 min and maintaining the final temperature for 15 min. After cooling, each sample was diluted in a volume of 25 mL with high-purity water (18 MΩ/cm) and then filtered with a 0.45-μm-pore size filter. Trace elements' quantifications were performed using an ICP-OES with an axial configuration (8,000 DV, Perkin Elmer) equipped with an ultrasonic nebulizer (Teledyne Cetac Technologies, Omaha, USA). Calibration standards were prepared and processed in the same way as samples up to the dilution step and were used to evaluate the concentration levels of trace elements (multi-element standard solution, CaPurAn, CPAchem, Stara Zagora, Bulgaria). The frequencies used for the determinations were as follows: Zn, 213.9 nm; Mg, 279.0 nm; Fe, 259.9 nm; Cu, 324.7 nm; Cd, 228.8 nm; and Se, 196 nm, through a specific accuracy analyzed with the use of ERM-BC21 (the European reference material).

2.4 Arabinoxylan content

The total (TOT-AX) and the water-extractable arabinoxylans (WE-AX) were measured on whole flours using the colorimetric methods reported respectively by Douglas (1981) and by Finnie et al. (2006) with some modification according to Romano et al. (2022). For each biological sample relative to each genotype, three technical replicates were done; TOT-AXs and WE-AXs were expressed as percentage of xylose using the following equations:

$$TOTAXs = \left(\frac{\text{mg}}{\text{g}} \right) = \frac{\text{absorbance (552 - 510)} - q}{m} \times 200$$

$$WEAXs = \left(\frac{\text{mg}}{\text{g}} \right) = \frac{\text{absorbance (552 - 510)} - q}{m} \times 200$$

where m is the slope and q is the intercept of the xylose standard curve.

2.5 β-Glucan

The β-glucan content was determined on whole flour using the β-Glucan Assay Kit (Mixed Linkage) (K-BGLU, Megazyme Ireland Ltd.). For each genotype, three technical replicates were analyzed.

2.6 Statistical analysis

Two different two-way ANOVA analyses ($\alpha = 0.05$) were used: one to assess the effect of genotype and year along with their interaction, and one to assess the effect of subspecies and year and their interaction as well. Mineral element data were log-transformed to ensure ANOVA assumptions. Significant differences were evaluated using Tukey's HSD (honestly significant difference) test ($\alpha = 0.05$). To evaluate the contribution of every variable to the total variance, the percentage of their sum of squares on the total was calculated.

Statistical analyses and plots of contents of mineral elements, arabinoxylans, and β -glucan were made with R using the packages *Rmisc*, *Agricolae*, and *ggplot2* (Wickham, 2016; Mendiburu, 2021; Hope, 2022; R Core Team, 2022).

2.7 Characterization of different genotypes at juvenile stage

2.7.1 Plant growing conditions

Seeds of the different genotypes were allowed to germinate in aeroponics in the dark at 28°C for 4 days. Uniform seedlings were selected and transferred in hydroponic culture in plastic pots (six seedlings per pot), containing 2 L of a nutrient solution (Quagliata et al., 2023). Plants were grown under controlled conditions with a day/night cycle of 16 h/8 h at 28°C/20°C air temperature, 80% relative humidity, and 200 $\mu\text{E m}^{-2} \text{s}^{-1}$ light intensity. After 5 days from sowing, plants were harvested and analyzed.

2.7.2 Determination of chlorophyll level

At harvest, chlorophyll level was determined on the youngest fully expanded leaf using a non-destructive portable apparatus, the Soil Plant Analysis Development (SPAD-502 Plus, Konica Minolta, Osaka, Japan). The values are provided as SPAD units.

2.7.3 Analysis of root morphological traits

Five days after sowing, the root apparatus of each genotype was excised from the stem and analyzed using the software WinRHIZO (Regent Instruments Inc., Quebec, Canada) and an Epson scanner as described by Quagliata et al. (2023).

2.7.4 Visualization of iron deposits in roots using the Perls stain

The localization of ferric iron (Fe^{3+}) in root tissues of the different genotypes was performed as described by Frittelli et al. (2023). Briefly, excised roots were incubated with a 2% $\text{K}_4[\text{Fe}(\text{CN})_6]$ and 2% HCl solution for 15 min at room temperature. Localization of Fe^{3+} was observed with a Zeiss (Oberkochen, Germany) Axioscop II microscope equipped with an epifluorescence illuminator (HBO50/AC BP 390-420 exciter filter and LP 450 barrier filter).

2.7.5 Statistical analysis relative to roots measurements

The results were presented as mean $\pm$ standard deviation (SD) of three independent biological replicates ($n = 3$). All data were

statistically analyzed by one-way ANOVA with Student's t-test at $p < 0.05$ by using the statistical software CoStat (version 6.45).

The hierarchical clustering was performed on the datasets by complete method and Euclidean distance measurement in R (R Core Team, 2022).

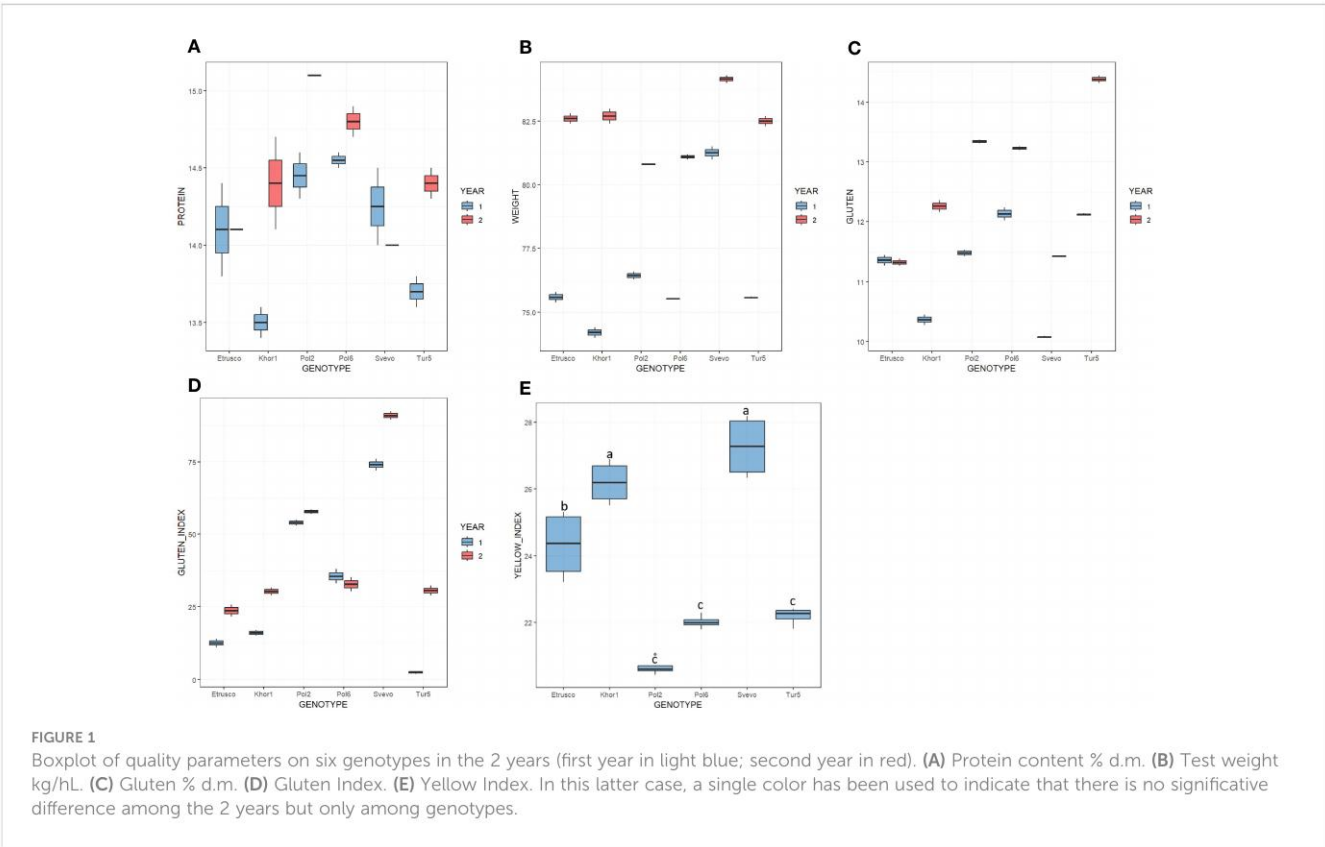
3 Results

3.1 Technological characterization of the tetraploid genotypes

The qualitative parameters summarized in Figure 1 and Supplementary Table 1 were evaluated in two growing seasons. In the first year, test weight values ranged from 74.2 kg/hL to 81.3 kg/hL, whereas, in the second year, from 80.8 kg/hL to 84.2 kg/hL. In both seasons, Svevo was the genotype with the highest value of test weight, whereas no significant difference was observed among the accessions of *T. turgidum* ssp. *turanicum* and *polonicum*. In the 2 years, the protein content varied between 13.5% and 15.1% dry matter. ANOVA analysis did not highlight significant differences between the two growing seasons and between the different genotypes in this regard. The gluten amount varied in the range from 10.1% to 14.4% and, in general, was higher in the second year except for Etrusco, which had similar values in the two growing seasons. Moreover, the Gluten Index was influenced by the growing season and showed a higher value in the second year for all accessions, except for Pol6 that showed a slight decrease. In detail, during the 2018–2019 season, the Gluten Index range varied from 3% in Tur5 and reached 74% in Svevo, whereas, during 2019–2020, it varied from 24% in Etrusco up to 91% in Svevo. Among all accessions analyzed, only Tur5 presented a strong difference in Gluten Index between the two growing seasons analyzed. The ANOVA showed for all qualitative parameters, except for the yellow index, a statistically significant influence ($p < 0.05$) by both genotype and year under study. Tukey's *post-hoc* test was performed on both variables (genotype $\times$ year). In particular, for the yellow index, ANOVA analysis highlighted significant differences among accessions but not for growing season (Figure 1E). Svevo and Khor1 presented the highest value, whereas Pol2, Pol6, and Tur5 presented the lowest.

3.2 Rheological properties of semolina

The alveographic P/L values obtained from semolina of the tetraploid accessions (except Khor1) were lower compared with that of Svevo in both growing seasons due to the reduced tenacity (P) and increased extensibility (L) of dough (Table 2). The values of Svevo and Khor1, on the other hand, showed a reduced extensibility at the expense of a better P/L value in the 2 years (1.66 and 1.12, respectively). In detail, the P/L ratio is lower in the second growing season and ranged from a minimum of 0.32 (Tur5 in the second year) to a maximum of 2.63 (Svevo in the first year). The W parameter showed more homogeneous values during the two crop



years from a minimum of 91 (Etrusco, second year) to a maximum of 246 (Svevo, second year) (Table 2).

3.3 Pasta evaluation

Spaghetti, the most common pasta shape worldwide, was evaluated for important quality parameters for consumers such as size, cooking time, and cooked texture (Table 3). The spaghetti diameter, which influenced the cooking time of the pasta, showed an increasing value in the second year with Svevo and Khor1 having the highest values. In the first year, the results showed no differences in

the optimal cooking times among genotypes. In the second year, however, this parameter showed a higher value for Svevo and Tur5 and a general decrease compared with that in the previous season. The looseness had differences among accessions in both years with lower values during the second season. Etrusco and Svevo showed the highest value (80) in the first year, followed by Pol2 and Tur5 (75). Differently, Tur5 and Khor1 had the best looseness (70) in the second season, followed by Svevo and Etrusco (60). The bulkiness showed a decrease during the second years with Khor1 presenting the highest value (55), whereas the other accessions showed the same value (50). In general, the parameters used for pasta evaluation were better in the first year than in the second for all the genotypes.

TABLE 2 Rheological parameters obtained from semolina analyzed with Chopin’s alveograph of accessions of *Triticum* subspecies.

Accession code	P		L		W(J.10 ⁻⁴)		P/L	
	2018–2019	2019–2020	2018–2019	2019–2020	2018–2019	2019–2020	2018–2019	2019–2020
Etrusco	60	50	65	71	117	91	0.92	0.7
Pol2	57	52	78	95	134	145	0.73	0.55
Pol6	52	42	80	65	127	86	0.65	0.65
Tur5	46	43	107	133	110	124	0.43	0.32
Khor1	n.a.	64	n.a.	57	n.a.	114	n.a.	1.12
Svevo	129	108	49	65	239	246	2.63	1.66

Data relative to Khor1 are available for the second year only. n.a., not available.

TABLE 3 Pasta values of three subspecies of *Triticum turgidum*.

Accession code	Spaghetti diameter (mm)	Optimal cooking times		Stickiness		Firmness		Bulkiness	
		2018–2019	2019–2020	2018–2019	2019–2020	2018–2019	2019–2020	2018–2019	2019–2020
Etrusco	1.52–1.58	9'40"	6'45"	80	70	80	60	75	50
Pol2	1.55–1.68	9'30"	6'40"	65	50	75	55	65	50
Pol6	1.54–1.58	9'35"	6'30"	70	50	70	50	75	50
Tur5	1.50–1.60	9'30"	7'30"	70	50	75	70	70	50
Khor1	1.65–1.72	n.d.	6'15"	n.d.	75	n.d.	70	n.d.	55
Svevo	1.62–1.66	9'40"	8'45"	70	50	80	60	70	50

Data relative to Khor1 are available for the second year only. n.d., not determined.

3.4 Characterization of mineral content in tetraploid genotypes

The analysis of the micro-elements (Zn, Fe, Cu, Cd, and Se) and macro-elements (Mg) was performed using the acid digestion method coupled with the ICP-OES technique on the different tetraploid accessions listed in Table 1; Svevo, Aureo, and Senatore Cappelli were used as reference genotypes. Results analyses are reported in Figure 2, Supplementary Figures 1, 2, and Supplementary Tables 2, 3. The mineral content was higher during the second year in which showed a higher range of variability among genotypes. Only iron showed an opposite trend: it presented higher concentrations either in the genotypes or greater variability in the first year than in the second. Table 4 reports the percentages of the sum of squares of each variable of the two ANOVA models. The first ANOVA analysis, which considered single accessions, highlighted significant effects on the content of each mineral by genotype, year, and interaction between them. Se

was the only element whose variance was mainly explained by genotype. The variances of Zn and Cd were mainly explained by the interaction between genotype and year. Lastly, the variances of Mg, Fe, and Cd were mainly explained by year. The second ANOVA analysis (Table 4, Supplementary Figure 1), which considered the different subspecies *T. durum*, *T. turanicum*, and *T. polonicum*, highlighted significant differences on the content of Cd and Se in three subgroups. The first was significantly higher in durum and Khorasan wheats than that in the Polish wheats, whereas the second was significantly higher in *T. polonicum* and significantly lower in *T. turanicum* than that in the others. There were no significant differences among subspecies for the other minerals. Zn, Fe, and Cd showed significant effects of the interaction between genotypes and year. For all the minerals, the effect of the year among subspecies was significant. Taking into consideration individual accessions, during the first growing season, the only mineral whose concentration did not show changes among genotypes was Zn; whereas, during the second year, all minerals showed a different

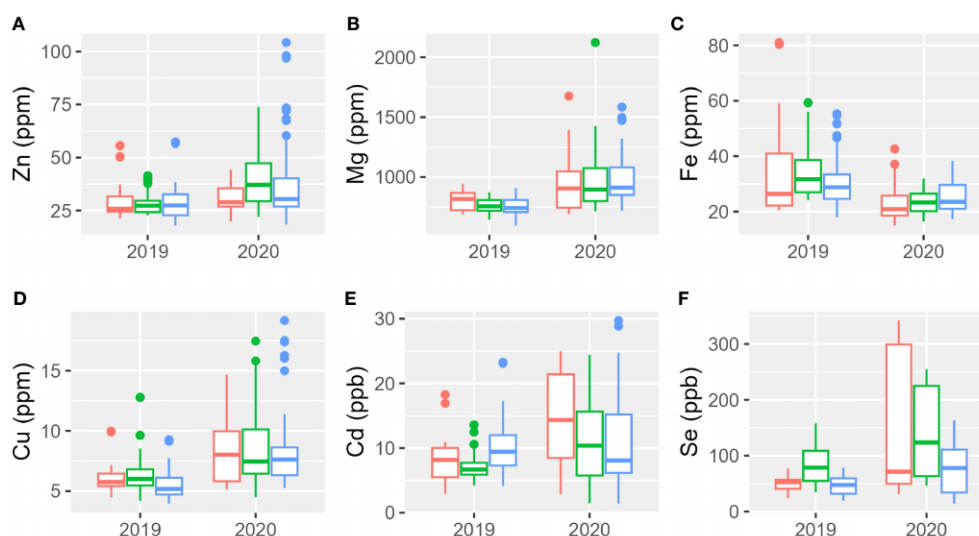


FIGURE 2
Boxplot of mineral elements content of the three subspecies studied in 2 years. In red, *T. durum*; in green, *T. polonicum*; and in blue, *T. turanicum*. (A) Zinc; (B) magnesium; (C) iron; (D) copper; (E) cadmium; (F) selenium.

TABLE 4 Minerals content of the three subspecies of *Triticum turgidum*.

Variable	Transformation	Sums of Squares 1				Sums of Squares 2			
		Genotype	Year	Genotype × Year	Residual	Subspecies	Year	Subspecies × Year	Residual
Zn	log	23.92***	12.88***	24.10***	39.09	1.45 ^{ns}	12.88***	2.29*	83.36
Mg	log	11.19***	30.52***	16.63***	41.64	0.12 ^{ns}	30.52***	1.15 ^{ns}	68.19
Fe	log	12.23***	22.72***	10.8***	54.23	0.28 ^{ns}	22.72***	2.14*	74.85
Cu	log	19.9***	27.52***	13.94***	38.61	1.13 ^{ns}	27.52***	0.89 ^{ns}	70.44
Cd	log	34.98***	3.22***	35.52***	26.26	2.86**	3.22**	3.65**	90.25
Se	log	58.6***	12.06***	21.70***	7.63	14.79***	12.06***	0.73 ^{ns}	72.40

Percentage of the sum of squares of each variable and of the residuals for the micro- and macro-elements content. Model 1 takes into consideration genotype, whereas model 2 takes into consideration subspecies. *p< 0.05, **p< 0.01, ***p< 0.001, and ^{ns}p > 0.05.

accumulation among genotypes (Supplementary Table 3). During 2018–2019, Khor1 had the lowest accumulation of Zn, whereas Tur5 had the highest accumulation. The highest accumulation of Mg occurred during the second growing season where Senatore Cappelli showed a lower accumulation in this mineral. Higher values were found in Pol2156>Khor1>Aureo>Tur21>Svevo. In the first year, the lowest accumulation of iron was found in Tur21, whereas the highest accumulation was found in Senatore Cappelli>Pol2>Pol304. In the second year, on the contrary, Senatore Cappelli showed the second lowest Fe accumulation, whereas Aureo>Tur26>Tur5 exhibited the highest. Senatore Cappelli is also characterized by a high accumulation of Cd and a low accumulation of Cu. Cd showed a high accumulation in Tur5, a low accumulation in Pol2. Finally, in both growing seasons, the accumulation of Se was lowest in Tur5 and highest in Pol304.

3.5 Characterization of Arabinoxylan and β-glucan content in tetraploid genotypes

Results relative to the evaluation of total (TOT-AX) and extractable (WE-AX) arabinoxylans are shown in Figure 3 and

Supplementary Table 4. Total arabinoxylan content ranged from 3.84% (w/w) to 5.88% (w/w); the water-extractable content ranged from 0.34% (w/w) to 0.93% (w/w); the β-glucan content ranged from 0.21% (w/w) to 0.50% (w/w). Both β-glucan and WE-AX contents were significantly higher in 2019. Table 5 reports the percentages of the sum of squares of each variable of the two ANOVA models. Considering the single accessions, the main contribution to the differences in WE-AX and β-glucan content was due to genotype, whereas TOT-AX content was mostly influenced by genotype-by-year interaction, in addition to by genotype. In both growing seasons, Tur26 showed the highest WE-AX content, whereas Senatore Cappelli and Pol304 were among the genotypes that accumulated the highest amount of TOT-AX and β-glucan, respectively (Supplementary Table 3). Differently, considering the second ANOVA model, only for TOT-AX and β-glucan, there were significant effects for the subspecies (Supplementary Figure 3), and, only for WE-AX and β-glucan, there were significant effects for the year (Supplementary Figure 4). No significant effects were found for the interaction subspecies × year. In both growing seasons, durum wheats showed the highest content of β-glucan and TOT-AX, whereas there were no significant difference among the subspecies for the WE-AX content.

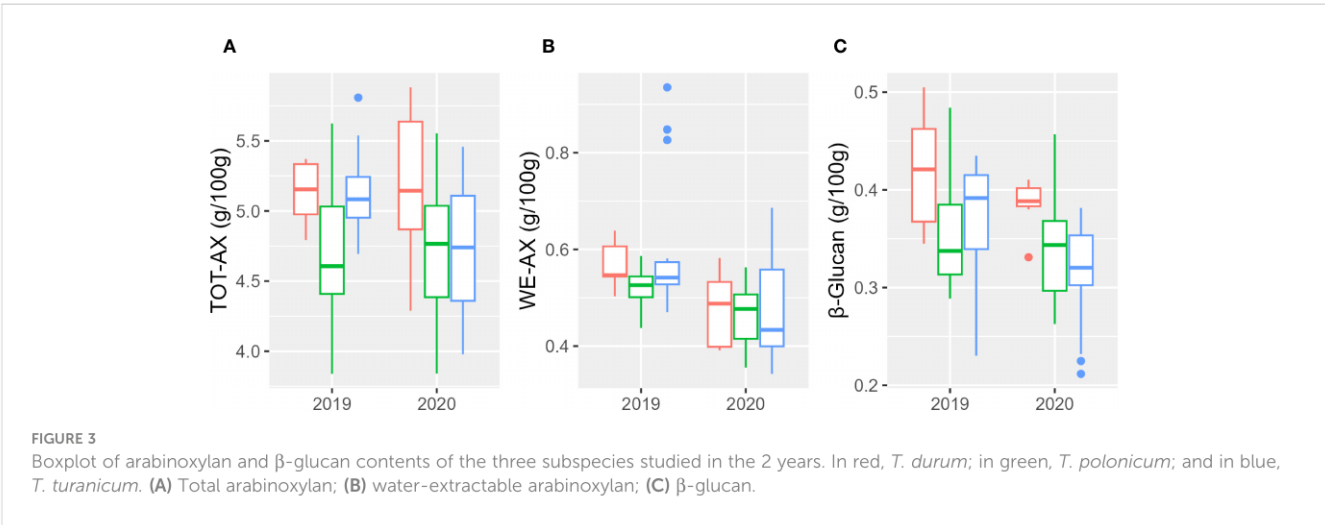


TABLE 5 Fiber content of the three subspecies of *Triticum turgidum*.

Variable	Sums of Squares 1				Sums of Squares 2			
	Genotype	Year	Genotype × Year	Residual	Subspecies	Year	Subspecies × Year	Residual
WE-AX	66.89***	18.31***	10.42***	4.35	5.15 ^{ns}	18.31***	1.55 ^{ns}	74.97
TOT-AX	38.85***	2.25***	30.31***	28.57	12.33**	2.25 ^{ns}	4.70 ^{ns}	80.70
β-Glucan	74.70***	7.17***	7.99***	10.11	15.75**	7.17*	1.62 ^{ns}	75.43

Percentage of the sum of squares of every variable and of the residuals for the fiber content. Model 1 (Sum of Squares 1) takes into consideration genotype, whereas model 2 (Sum of Squares 2) takes into consideration subspecies. *p< 0.05, **p< 0.01, ***p< 0.001, and ^{ns}p > 0.05.

3.6 Characterization of different genotypes at juvenile stage

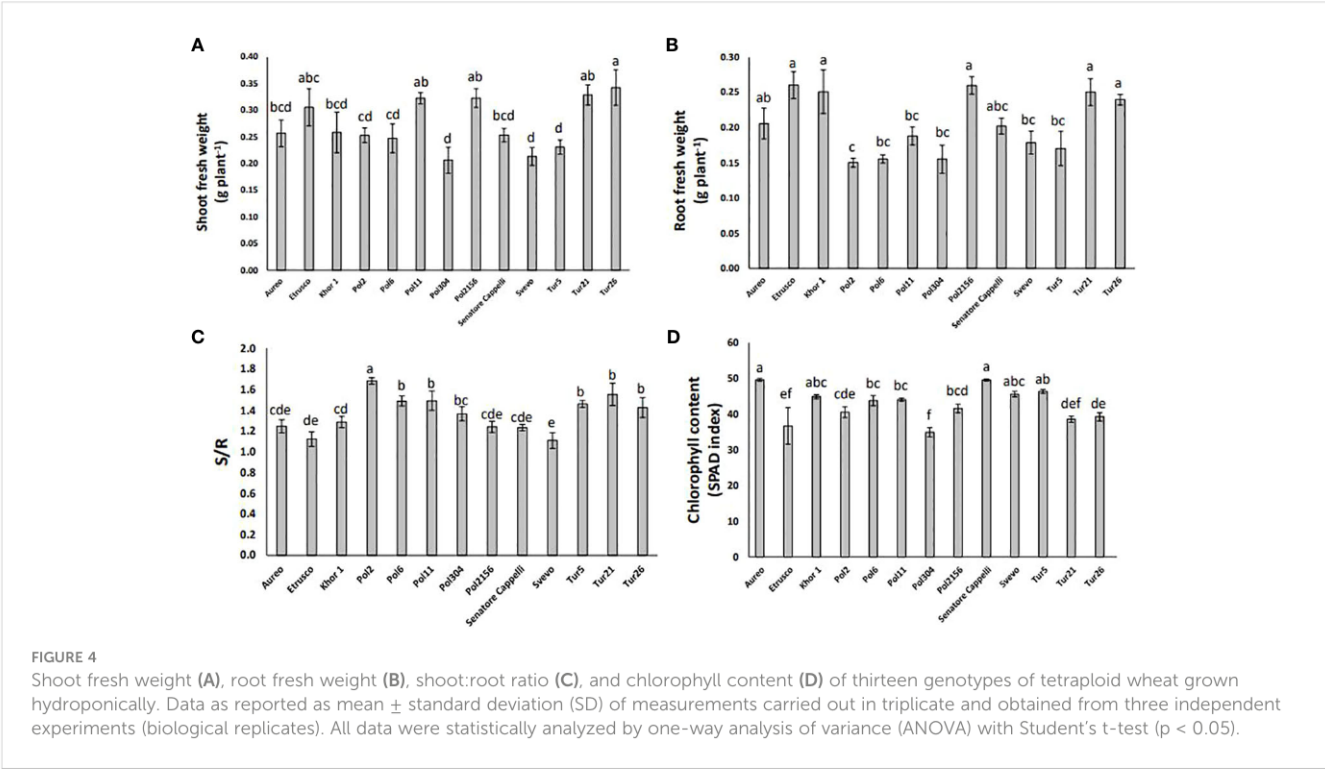
3.6.1 Plant growth rate and chlorophyll content

The plant growth rate was measured as fresh biomass production of both shoots and roots. The genotypes Pol304, Svevo, and Tur5 showed the lowest shoot biomass production, whereas the genotype Tur26 showed the highest one (Figure 4A). In particular, in the genotypes Pol304, Svevo, and Tur5, shoot biomass production was reduced by 40%, 38%, and 33%, respectively, as compared with that in the genotype Tur26 (Figure 4A). On the other hand, the genotype Pol2 showed the lowest root fresh biomass production (Figure 4B). The shoot-to-root ratio (S/R) plays a crucial role in a plant’s overall growth and development, being directly linked to how a plant allocates its resources, such as nutrients, water, and carbohydrates. The genotypes Svevo and Pol2 showed the lowest and the highest S/R, respectively (Figure 4C). Finally, measuring chlorophyll level in leaves of all different genotypes, there was no large variation in SPAD units

across this population. In particular, the genotype Pol2156 reached the lowest value, which was 16% lower with respect to Aureo and Senatore Cappelli, the genotypes showing the highest chlorophyll level (Figure 4D).

3.6.2 Root morphological traits

Root system architecture is crucial for exploring soil and acquiring resources, as well as for crop adaptation to stress and productivity. Using WinRHIZO scanning equipment, a set of morphological traits was assessed, including length, volume, diameter, surface area, and number of tips of the root systems (Figure 5). To facilitate data discussion and identify major clusters across all different genotypes, hierarchical clustering was performed on the morphological traits dataset by complete method and Euclidean distance measurement (Figure 6). Results showed that both Tur21 e Tur26 clustered together and showed the highest values of all morphological traits (Figure 6). On the other hand, in Svevo, all morphological traits reached the lowest values, although Svevo showed the most balanced S/R (Figure 6).



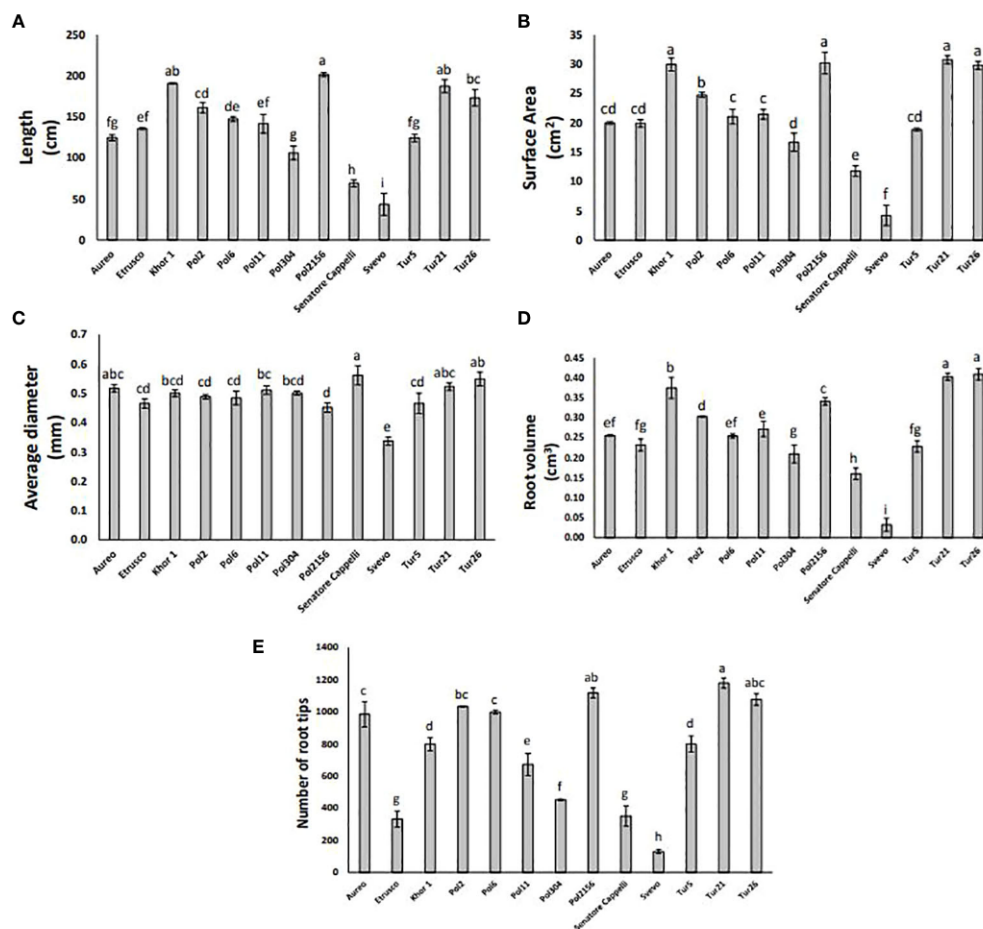


FIGURE 5

Root length (A), root surface area (B), root average diameter (C), root volume (D), and number of root tips (E) of the thirteen tetraploid wheat genotypes at 9 days after sowing. Data as reported as mean $\pm$ standard deviation (SD) of measurements carried out in triplicate and obtained from three independent experiments (biological replicates). All data were statistically analyzed by one-way analysis of variance (ANOVA) with Student's *t*-test ($p < 0.05$).

3.6.3 Visualization of iron deposits in root tissues

The distribution and/or localization of ferric deposits in the root tissue of different durum wheat genotypes were evaluated using a very sensitive Fe-specific histochemical procedure. Obtained images showed that iron deposits, indicated by blue staining, were mainly localized at the level of the central zone of the primary root and the root hairs present in the same zone (Figure 7). Interestingly, the highest level of accumulation was observed for Pol2, as evidenced by the more intense blue staining than that obtained with the other genotypes (Figure 7).

4 Discussion

On the basis of the growing interest of consumers in wheats present before the Green Revolution, collectively named “ancient wheats,” parallel research is increasing to analyze them more in detail both from the nutritional and technological points of view (Cabas-Lühmann et al., 2023; Roumia et al., 2023).

In this paper, we have analyzed accessions of the *T. turgidum* group, including the subspecies *T. durum* or durum wheat, *T. polanicum* or Polish wheat, and *T. turanicum* or Khorasan wheat, grown in the same environment for 2 years, in terms of technological and nutrition-related aspects. Moreover, the same accessions have been hydroponically grown to test root morphology in order to possibly associate some of the characteristics that have been identified.

As regards the technological analyses, although protein and gluten contents were comparable among the accessions and in the 2 years and coherent with the average Italian cultivars (De Santis et al., 2017), the modern variety Svevo, as expected, had the best performance followed by Khor1. It is important to underline that Khor1 corresponds to the local field cultivation of KAMUT® brand wheat; thus, it was not possible to use this name for locally produced kernels because they were obtained out of the official KAMUT® food chain. KAMUT® brand wheat has been selected also because of its better yield and technological performance; thus, it is expected that some of the qualitative parameters here measured are very good for Khor1 compared with that for other Khorasan or Polish wheats.

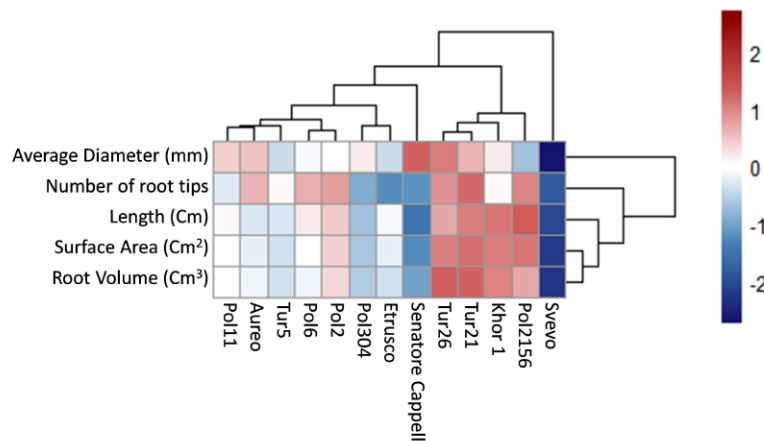


FIGURE 6

Heatmap representing hierarchical clustering of root traits dataset of thirteen tetraploid wheat genotypes by complete method and Euclidean distance measure. The color bar depicts the gradient of values of roots traits.

The test weight, a physical quality characteristic desirable because of its positive influence on market grade and price (Troccoli and Di Fonzo, 1999), showed a significant difference during the two crop years, with the first with significantly lower values probably due to three-fold more rainfall than in the second year. As reported in Kuzmanović et al. (2021), the 2019 season, at Nello Lupori experimental farm in Viterbo, was characterized by a high amount of rainfall and lower temperatures during the grain filling and the authors hypothesized that these conditions led to greater plant tiller number with less seed weight (meteorological data relative to the same location and years).

The yellow index was highly different among accessions, but not between the two growing seasons. This also is expected on the basis of the great heritability of this character (Colasuonno et al., 2019). In this case, also, Svevo and Khor1 showed the highest value.

The micro- and macro-nutrient amounts were measured because of their great nutritional importance (Khalid et al., 2023) and for their healthy beneficial effects such as antioxidant, anti-cancer, and anti-viral properties (Welch and Graham, 2004; Hansch and Mendel, 2009; Abbaspour et al., 2014). Furthermore, there is a common belief that ancient wheats contain a higher amount of these minerals (Shewry et al., 2016; Lovegrove et al., 2020; Shewry et al., 2020).

In our case, by considering the single accessions, the ANOVA analysis indicated that selenium variance was mostly explained by the genotype. In contrast, Lyons et al. (2005) reported that the amount of selenium in the kernels of wheat cultivars from Australia and Mexico is primarily determined by its availability and concentration in the soil. In fact, although higher concentrations of selenium, zinc, lithium, magnesium, and phosphorus were found in hulled wheat (*Triticum spelta* L. and *Triticum dicoccum* Schrank) grown together with modern bread wheats (Piergiovanni et al., 1997), several studies found no evidence for genetic variability among wheat cultivars for selenium accumulation in grain (Grela, 1996; Tveitnes et al., 1996; Zhao et al., 2009; Souza et al., 2014). However, a considerable genotypic variation and capacity in accumulating selenium, at least in root and shoot tissues, by four

different tetraploid wheats, have been recently demonstrated (Coppa et al., 2023). In particular, the different selenate uptake ability was mainly explained by the differential expression of genes encoding the two high-affinity transporters (TdSultr1.1 and TdSultr1.3), which are involved in the primary uptake of sulfate (and selenate) from the growth medium (Coppa et al., 2023). In our work, by considering the different subspecies, the selenium concentration was lower in the Khorosan wheat in both growing seasons, but the differences were only partially explained by subspecies, year, and subspecies $\times$ year.

Our analyses highlighted that the accumulation of zinc among different accessions is mostly influenced by the genotype and interaction between genotype and year. This observation agrees with previous studies that reported a wider genetic variation for iron and zinc concentrations in diploid and tetraploid species such as *T. monococcum*, *T. turgidum* ssp. *dicoccoides*, *T. boeoticum*, *Aegilops tauschii*, *T. spelta*, *T. turgidum* spp. *polonicum*, and some landraces (Cakmak, 2007; Velu et al., 2014).

The concentration of cadmium among different accessions is mostly influenced by the genotype and interaction between genotype and year, whereas, by considering the different subspecies, its concentration was significantly higher in durum and Khorasan wheat. This heavy metal, in recent years, has become one of the major and widespread soil pollutants due to intensive agricultural practices, unsustainable industrialization, and urbanization. Being highly toxic for plants, animals, and humans (Sarwar et al., 2010), there is growing interest in the selection of varieties that accumulate low cadmium levels. Cadmium accumulation in plants is determined by several genes that regulate mechanisms underlying its uptake, translocation, and accumulation (Tao and Lu, 2022). A class of metal transporters called Natural Resistance-Associated Macrophage Proteins (NRAMPs) was first reported in the model plant *Arabidopsis thaliana* involved in cadmium transport. Recently, three genes (*TpNramp3*, *TpNramp5*, and *TtNramp6*) encoding plasma membrane proteins involved in cadmium concentration and accumulation in the whole plant were found in *T. polonicum* and

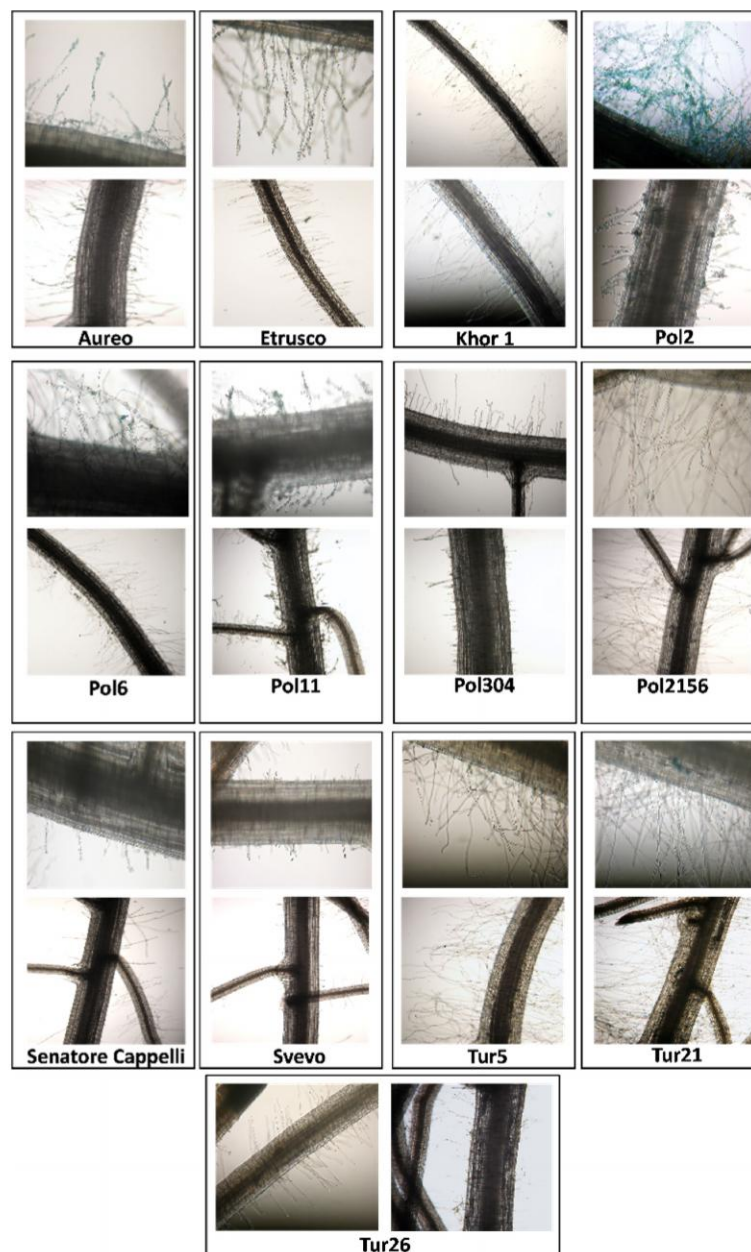


FIGURE 7
Visualization of iron deposits in roots of thirteen tetraploid wheat genotypes, using Perl's staining technique.

T. durum. Furthermore, Maccaferri et al. (2019) found that the modern durum wheats possessing the non functional copy of the *TdHMA3-B1A* gene accumulated higher amount of cadmium. Together, these results suggest that there are allelic variants in these genes in the different subspecies that regulate cadmium accumulation.

In general, by considering the different subspecies, the effect of the year among the three groups of subspecies was significant for all the minerals.

Finally, the results showed that the most interesting accessions were the following: Pol2, which accumulated the largest amount of zinc, magnesium, iron, and copper and had the lowest concentration of cadmium during the two seasons; Tur5, which

showed the greatest accumulation of zinc, copper, and cadmium and the lowest accumulation of selenium; Senatore Cappelli, which accumulated a low amount of magnesium and copper but a high amount of cadmium; and Pol304, which showed high accumulation of iron and selenium. Interestingly, Pol2 showed the highest level of iron accumulation in root tissues, most likely suggestive of a significant apoplastic iron pool (Bienfait et al., 1985), but its iron concentration in seeds indicates a low relative efficiency of the “root-to-seed” iron transfer. Nutrient acquisition by roots is a key component of nutrient use efficiency (NutUE), but it is not the only one at least for iron. Indeed, it has been demonstrated that although the supply of iron from soil to the root apoplast should be adequate, the transport from the apoplast to the symplast is often impaired

(Mengel, 1994); thus, even if a plant shows high concentrations of iron at root level, it can, however, show iron deficiency symptoms (Kosegarten and Koyro, 2001). High amounts of extraplasmatic iron have been detected in the roots of plants grown in nutrient solution (Bienfait et al., 1985), because, in roots, iron is mainly present as apoplastic pool, and this iron is basically adhering to the outer surface of the epidermis, probably in a particular form, and does not contribute significantly to the nutrition of the plant (Strasser et al., 1999). Furthermore, Grusak and Cakmak (2005) demonstrated that, even at high iron supply, the concentration of iron in shoot and seed could remain relatively low, likely due to limited phloem mobility. Therefore, the promising lines rich in micro- and macro-nutrients and poor in toxic elements such as cadmium identified in this study must be validated in multilocation trials. This study confirmed that a large variation in the concentrations of microelements is present among different accessions and is not solely intra-subspecies, because the environment and soil nutrient status are known to significantly affect these traits (Shewry et al., 2020).

The fibers (that include β -glucan and arabinoxylan) influence the quality of pasta and bakery products, due to their high capacity to bind the water present in the dough and to increase its viscosity. Furthermore, they have numerous beneficial physiological effects for humans, i.e., reduction of blood cholesterol, attenuation of blood glucose levels, and elevated prebiotic activity (Botticella et al., 2021). We found that, by considering the single accessions, the main contribution to the differences in WE-AX and β -glucan content was due to genotype, whereas TOT-AX content was mostly influenced by genotype-by-year interaction, in addition to by genotype. These results agree with the previous studies reported by Marion and Saulnier (2020). In general, Lovegrove et al. (2020), by analyzing 39 wheat cultivars dated between 1790 and 2012, showed that the environment affects the accumulation of health-related components, among which arabinoxylans, to a great extent. Instead, considering the subspecies, only for TOT-AX and β -glucan, there were significant effects, and, only for WE-AX and β -glucan, there were significant effects for the year. No significant effects were found for the interaction subspecies $\times$ year. In both growing season, durum wheats showed the highest content of β -glucan and TOT-AX, whereas there are no difference among the subspecies for the WE-AX content.

Root system plays a crucial role in plant fitness as roots are responsible for the efficient uptake of water and mineral nutrients. Therefore, root development and morphology were related to quality parameters (in terms of yield and technological and nutritional quality). In particular, the S/R is a measurement of the amount of shoot biomass (growth function) compared with the amount of root biomass (supportive function) and is generally maintained within a certain balance that is characteristic of the species. However, in this study, only the modern variety Svevo showed a well-balanced S/R, thus an optimal distribution of resources between the aboveground and belowground portions of the plant. This allocation should enable more efficient utilization of water and nutrients. On the other hand, the lowest

values of all measured root morphological traits were observed in Svevo. Tur21 and Tur26 clustered together and showed the highest values of all morphological traits, as well as Pol2 and Pol6 clustered together and showed an increase in all roots morphological parameters compared with Svevo. Nutrient uptake in plants is, in fact, closely related to root morphology. In particular, the length and surface area of plant roots, as well as the number of root hairs, are directly related to their capacity to absorb nutrients. Therefore, although Pol2 had a lower root biomass, the particular morphology of the roots is more efficient for nutrient uptake, as evident from the greater accumulation of micro- and macro-nutrients.

In conclusion, the two accessions of Polish wheat Pol2 and Pol6 have a potential good technological quality. Moreover, Pol2 is rich in health-related micro- and macro-nutrients and poor in toxic ones. Thus, these two accessions may be taken into consideration for the development of new varieties.

More information about dynamics of iron distribution in shoots and seeds is crucial to guide the exploitation of these findings to possibly improve the nutritional value of grains by increasing iron content. Thus, further experiments will explore the diversity of these wheats, with special interest in their relative efficiency of the “root-to-shoot” and “shoot-to-seed” iron transfer.

Finally, these results confirm that it is not possible to draw general conclusions about the difference between old and modern wheats, but rather a case-by-case approach should be taken into consideration, by including both the different genotypes and growing conditions.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Author contributions

DL, FS, and SM: conceptualization; SP and MB: analysis of mineral and fiber content; AC: quality-related analysis, GQ and SA: root characteristics analyses, SP, MB, AC, GQ, SA, FS, and SM: writing the original draft; SP, MB, AC, GQ, SA, DL, FS, and SM: review and editing. All authors contributed to the article and approved the submitted version.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fpls.2023.1269212/full#supplementary-material>

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After this work, we shifted our focus on Sicilian tetraploid conservation varieties. In Sicily, they are still cultivated, especially in marginal areas, and they have an important impact on the local economy. Most of the varieties are durum wheat, but there is also one Khorasan wheat variety, Perciasacchi, and different Rivet wheat varieties (Bivona, Ciciredda, Paola).

We started the characterization of Sicilian tetraploid conservation varieties analysing the intra- and inter- varietal variability using gluten protein composition. In collaboration with Consorzio di Ricerca Gian Pietro Ballatore, CREA-DC of Palermo and CREA-DC of Tavazzano, we collected 51 accessions belonging to 22 Sicilian conservation varieties (most of the varieties registered to the National Catalogue of agricultural species).

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4 Chapter II - Characterization among and within Sicilian Tetraploid Wheat Landraces by Gluten Protein Analysis for Traceability Purposes

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Article

Characterization among and within Sicilian Tetraploid Wheat Landraces by Gluten Protein Analysis for Traceability Purposes

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Abstract: The criteria of “Distinctness, Uniformity and Stability” as well as a high “overall quality index” are used to register the Italian modern varieties to the national register. Differently, local conservation varieties can be certified under different EU Directives that facilitate, as an overall objective, the preservation of biodiversity and the containment of genetic erosion. In recent years, products derived from ancient grains are perceived to be healthier and more sustainable by consumers, especially in Italy, with consequent higher market prices. The ancient tetraploid wheat varieties registered in the national register of conservation varieties amount to 28, 24 of which are Sicilian. They are supposed to have wide genetic variability compared to modern ones, making them vulnerable to fraud because they are difficult to trace. It is therefore important to have tools able to discriminate between autochthonous Sicilian varieties. This can be completed by gluten proteins composition, which also provides information on the technological properties of derived products. Fifty-one accessions belonging to twenty-two ancient varieties of Sicilian tetraploid (mostly durum) wheat were analyzed. Although wide intra-accession and intra-varietal variability measurements were assessed, the gliadin pattern of bulks of seeds belonging to each variety was discriminatory. Moreover, differences in technological attitudes were found between landraces. This paves the way to use gluten protein patterns for traceability, allowing local farmers and producers to valorize their products and assure consumers regarding the transparency of the entire supply chain.

Keywords: durum wheat; landraces; traceability; gliadin; glutenin subunits



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1. Introduction

Wheat is one of the most cultivated crops in the world and provides a major source of nutrition globally. About 95% of the wheat grown worldwide is hexaploid bread wheat (*Triticum aestivum* L., $2n = 6x = 42$, genome AABBDD), whilst the remaining 5% is mainly represented by durum wheat (*Triticum turgidum* ssp. *durum*, $2n = 4x = 28$, genome AABB) [1]. The latter is well-adapted to semiarid environments and is mostly grown in the Mediterranean regions and North America.

After Canada, the second largest producer of durum wheat in the world is Italy, with 3.9 million tons (average data of the period 2019–2023), and more than half of the production comes from the southern regions [2,3]. The primary role of Italy in the cultivation of durum wheat is partly attributable to the use of semolina to produce traditional foods of the Mediterranean diet. In particular, pasta is a highly popular durum wheat-based food product because of its convenience, versatility, sensory, and nutritional values [4]. According to Italian law (Art. 6—DPR 187/2001), it can only be made with semolina. The economic importance of the pasta industry has fueled the intense breeding work carried out in Italy since the beginning of the 20th century. The main durum wheat breeding programs were focused on the release of elite varieties with higher grain yield and improved protein composition, strictly related to dough and pasta quality traits [5–7].

The spread of modern varieties caused a drastic reduction in the cultivation of landraces and old cultivars, which are characterized by lower yield and technological performance and general late maturity coupled with higher stature. For these reasons, they were grown for years in marginal areas and their conservation was delegated to public research institutes and custodian farmers. However, in recent years, there has been an increasing interest in old and landrace wheats, collectively known as “ancient wheats”, especially in Italy, because the consumers perceive their products to be more healthy, “natural”, and sustainable compared to modern ones. To date, the literature has shown conflicting results regarding the superior health properties of old varieties and landraces. Several studies demonstrated that some of these accessions have a higher content of phenols [8–10], a higher concentration of minerals [11], and increased prebiotic carbohydrates [12] compared to elite varieties. Other studies supposed that their superior beneficial effects were not due to their characteristic of being “old” but that they are more related to genotype and growth conditions [13–17]. To prove the beneficial-promoting attributes of ancient varieties, further investigations are required, with standard analysis methods on multiple genotypes of old and modern wheat grown in replicated multi-site field trials [18].

Nevertheless, the old cultivars and landraces represent a precious resource of genetic variability for traits associated with tolerance to (a)biotic stress and adaptation to different pedoclimatic conditions and low-input farming systems [19–23]. These favorable alleles or QTLs can be introgressed in elite varieties.

The landraces and historical varieties are populations that do not fall within the regulatory standards that define modern varieties today. In Italy, since 2009, these varieties “which are naturally adapted to the local and regional conditions and threatened by genetic erosion” can be registered in the National Catalogue of agricultural species (Legislative Decree No. 149 of 2009, now repealed and replaced by Legislative Decree No. 20 of 2021). This decree has the general objective of safeguarding biodiversity and limiting genetic erosion in agriculture. Nowadays, 28 varieties of tetraploid wheat are registered in the national catalogue as conservation varieties, and 24 of these are Sicilian.

In Sicily, the agriculture sector is of fundamental importance for the island economy and for the development of rural communities. Tetraploid wheat, including both *Triticum turgidum* ssp. *durum*, ssp. *turgidum* and ssp. *turanicum*, the latter also known as Khorasan wheat, represents one of the most important crops in terms of cultivated area (272,405 ha) [2], and some landraces are still grown in the mountain areas, constituting an important resource for low-input farming in marginal areas [24]. Although the old varieties are still produced, conserved, and exchanged among local farmers, the preservation of grain purity is often difficult during harvesting and post-harvesting procedures. Indeed, the lack of traceability along the supply chain (from seed to end product) does not guarantee the production and marketing of certified grain, increasing the risk of fraud and putting farmers’ economic profits at risk.

In this context, many efforts have been undertaken to preserve old varieties (i.e., Sicilian landraces) in purity and their traditional end products, with the aim of avoiding commercial fraud. Different strategies have been used in the development of traceability methods. Fiore et al. [25] used Cluster Analysis and Principal Component Analysis

(PCA) obtained by single-nucleotide polymorphism (SNP) markers, together with agromorphological, phenological, and quality-related traits, to classify and evaluate Sicilian ancient wheat germplasm. The same authors demonstrated that the SNP panel represents an efficient tool for the genetic traceability of old wheat varieties and can help to elude commercial fraud, sustaining the economic profits of the farmers. Fiore et al. [26] used the same strategy with a wider germplasm collection, including 75 accessions of Sicilian landraces, achieving the same results. Similarly, Taranto et al. [27] dissected the genetic variation patterns of two large germplasm collections of “Timilia” and “Russello” using SNP genotyping. Other molecular markers, such as RFLPs and SSRs, were successfully used for the characterization of durum wheat germplasm collections, enabling variations and resources of landraces to be more accessible for exploitation [28–34]. More analyses were carried out by markers based on morphological descriptors, storage protein composition, digestibility of starch, and concentration of secondary metabolites, permitting discrimination between new elite varieties and landraces [8,35–40].

Among biochemical markers, prolamins constitute an efficient system for the traceability of ancient wheat varieties due to their polymorphic nature [41,42]. Prolamins are storage proteins characterized by high proline and glutamine contents and distinguished in polymeric alcohol-insoluble (glutenins) and monomeric alcohol-soluble (gliadins) fractions. Both fractions constitute gluten, a visco-elastic network responsible for the rheological and technological quality of dough. Glutenins confer strength and elasticity to dough [43–45] and are formed by polymers stabilized by intra- and inter-chain disulfide bonds. The reduction of these bonds releases the individual glutenin subunits that have been classified in high-molecular-weight (HMW-GS) and low-molecular-weight (LMW-GS) glutenin subunits, on the basis of their molecular weight in Sodium Dodecyl Sulphate-PolyAcrylamide Gel Electrophoresis (SDS-PAGE) (reviewed by [46,47]). Gliadins confer extensibility and viscosity to dough and have been classified in three groups, known as α/β -, γ -, and ω -gliadins, based on their decreasing mobility in electrophoresis at acid pH [48,49]. In particular, durum wheat technological quality is mostly due to the presence of B-type low-molecular-weight glutenin subunits, with many subunits encoded by the *Glu-B3* locus. Two major groups of LMW-GS, designed LMW-1 and LMW-2, associated with γ -42 and γ -45 gliadins, were identified and associated with poor and good technological quality, respectively. In addition, both groups of subunits include components controlled not only at the *Glu-B3* locus but also at the *Glu-A3* and *Glu-B2* loci [50]. In both group variants, differences regarding the presence or absence of subunits have also been identified [51]. Although it is definitively clear that the technological quality is due to LMW-2 rather than to γ -gliadins, it is still not clear yet if the association between the presence of this allelic form and quality is due to structural or quantitative differences [52].

Visioli et al. [53] used HMW-GS as markers to analyze the genetic purity of grains and flours marketed and labelled as monovarietal. This study was carried out on four different tetraploid wheat Sicilian landraces (Timilia, Russello, and Margherito, which are durum wheats, and Perciasacchi, which is a Khorasan wheat) and highlighted numerous cross-contaminations by the other local varieties of durum and bread wheat grown on the same farm. They indicated that HMW-GS analysis was a useful marker to trace the varietal correspondence and genetic pureness of grains.

In this work, the biochemical profiles of prolamin proteins were used to trace the varietal correspondence and gluten quality of 51 accessions derived from 22 Sicilian landraces and old durum/tetraploid wheat varieties obtained by different custodian farmers in two different growing seasons.

2. Results

2.1. Analysis of Gluten Protein

The electrophoretic separations of the gluten components present in the Sicilian varieties were analyzed with the aim to evaluate the presence of heterogeneity in each accession. The A-PAGE and SDS-PAGE analyses of the 40 *T. durum*, 2 *T. turgidum*, and 9 *T. turanicum*

accessions, listed in Table S1, were carried out along with five varieties used as controls. To characterize the presence of intra-accession variability, ten spikes (two kernels for each) and a bulk sample were studied for each accession. Both electrophoretic analyses showed heterogeneity (Table S2), thus indicating the presence of variability. The gliadin patterns showed greater differences than the glutenin ones and showed heterogeneity on 44 accessions of 51 studied (Table S2). Instead, five accessions (Bivona, Ciciredda, Gioia, and two accessions of Perciasacchi) had a homogeneous profile for both gliadins and glutenins. The analysis of bulk samples confirmed the heterogeneity among accessions of the same variety both for gliadin and glutenin profiles (Figures S1–S5).

To obtain a prolamins profile representative of each accession (here and after called “accession reference pattern”), all the kernels from ten spikes were pulled together. The electrophoretic analyses (Figures S1–S5) detected inter-accession polymorphisms except for Scorsonera, Faricello, and Castiglione Glabro, which were uniform. Of the three accessions of Timilia Reste Bianche, one presented a different profile for gliadins (TRB-4), while Timilia Reste Nere showed inter-accession heterogeneity for both gliadins and LMW glutenin subunits (LMW-GS). Ruscia (synonymous with Russello Ibleo) showed two different profiles for gliadins and LMW-GS. Four accessions of Bidì were analyzed and one of them (BID-1) showed different profiles for gliadins, HMW-GS, and LMW-GS. The main differences were probably due to the mixture of two landraces differing at *GluB1* (20x + 20y and 13x + 16y), *GliA1*, and *GliA2* loci. Nine accessions of Perciasacchi (Khorasan wheat) showed two different composition profiles for both gliadins and glutenins. In particular, PER-3, PER-6, PER-8, and PER-13 had the typical composition of HMW-GS (Bx6 + By8), while PER-9 presented the subunit Bx20 + By20. The other accessions presented a mixed profile of the previous ones. Moreover, morphological analysis of these materials (data unpublished) revealed that two main types of Perciasacchi are grown by custodian farmers in Sicily, differing in terms of flowering time and beak morphology, although the most common seems to be the type showing Bx6 + By8 HMW-GS.

To obtain a representative profile of each landrace, a pool of kernels from all the studied accessions (here and after called landrace reference patterns) were analyzed (Figure 1). The electrophoresis analyses permitted to distinguish a unique storage protein composition for each landrace for both gliadins and glutenins. Timilia Reste Bianche and Timilia Reste Nere showed the same gliadins pattern but different C-type LMW-GS. The only exceptions in which gliadins and glutenins failed to distinguish the unique profiles were for the landraces Bidì, Senatore Cappelli, and Capeiti 8, which showed similar profiles either for gliadins or glutenins.

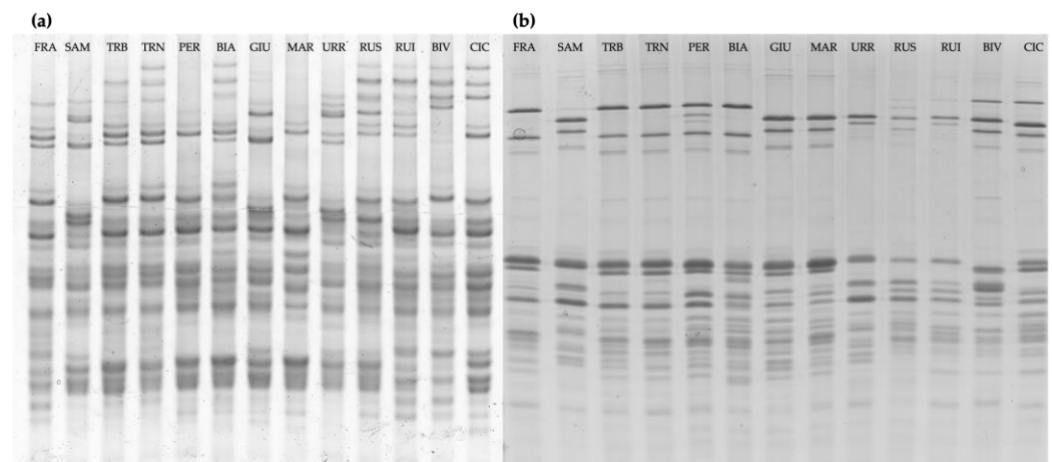


Figure 1. Cont.

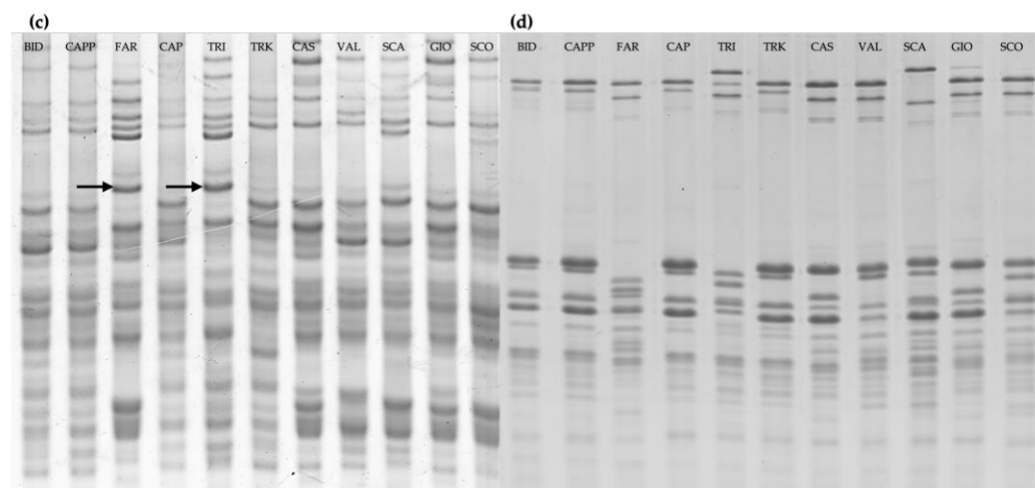


Figure 1. Electrophoretic separation of gluten proteins of the varietal bulks. **(a,c)** A-PAGE analysis of gliadins fractions. **(b,d)** SDS-PAGE analysis of glutenins fraction. FRA, Francesa; SAM, Sammartinara; TRB, Timilia reste bianche; TRN, Timilia reste nere; PER, Perciasacchi; BIA, Biancuccia; GIU, Giustalisa; MAR, Martinella; URR, Urria; RUS, Russello; RUI, Ruscia; BIV, Bivona; CIC, Ciciredda; BID, Bidì; CAPP, Senatore Cappelli; FAR, Faricello; CAP, Capeiti; TRI, Tripolino; TRK, Trinakria; CAS, Castiglione Glabro; VAL, Vallelunga; SCA, Scavuzza; GIO, Gioia; SCO, Scorsonera. The arrows indicate the γ -42 associated with the presence of LMW-1 in the varieties Faricello e Tripolino. All the other varieties show allelic forms of LMW-2 type **(a,d)**.

In Table 1, the different gluten allelic compositions in the studied accessions are listed. Since LMW-GS attribution to a specific allelic form is susceptible to errors due to several protein bands with similar electrophoretic mobilities, we decided to attribute to the LMW-1 or LMW-2 types on the basis of the presence, in the latter, of the slowest moving protein band, named 42K LMW-GS, which characterizes this allelic form, whereas its absence identifies the LMW-1 types [52].

Table 1. Details of the allelic composition of gluten genes of Sicilian landraces. HMW-GS coded by *Glu-A1* and *Glu-B1* loci are listed in sequence.

ID_CREA	Conservation Varieties	HMW-GS	LMW-GS Type	γ -Gliadins
FD-BIA-1	Biancuccia	null, 6 + 8	2	42
FD-BID-1	Bidì	null, 20x + 20y	2	45
FD-BID-2	Bidì	null, 20x + 20y	2	45
FD-BID-3	Bidì	null, 20x + 20y	2	45
FD-BID-5	Bidì	null, 20x + 20y	2	45
FD-BIV-1	Bivona	2*, 13 + 16	1	44
FD-CAP-1	Capeiti 8	null, 20 + 20	2	45
FD-CAS-2	Castiglione Glabro	null, 13 + 16	1	45
FD-CAS-3	Castiglione Glabro	null, 13 + 16	1	45
FD-CIC-1	Ciciredda	2*, 32 + 33	2	45
FD-FAR-1	Faricello	null, 13 + 16	1	42
FD-FAR-2	Faricello	null, 13 + 16	1	42
FD-FRA-1	Francesa	null, 6 + 8	2	45
FD-GIO-1	Gioia	null, 6 + 8/13 + 16	1	45
FD-GIO-2	Gioia	null, 13 + 16	1	45
FD-GIU-1	Giustalisa	null, 13 + 16	2	47
FD-MAR-1	Martinella	null, 13 + 16	2	45

Table 1. Cont.

ID_CREA	Conservation Varieties	HMW-GS	LMW-GS Type	γ -Gliadins
FD-PER-1	Perciasacchi	null, 6 + 8/20x + 20y	2	45
FD-PER-2	Perciasacchi	null, 6 + 8/20x + 20y	2	45
FD-PER-3	Perciasacchi	null, 6 + 8	2	45
FD-PER-4	Perciasacchi	null, 6 + 8/20x + 20y	2	45
FD-PER-6	Perciasacchi	null, 6 + 8	2	45
FD-PER-7	Perciasacchi	null, 6 + 8/20 + 20	2	45
FD-PER-8	Perciasacchi	null, 6 + 8	2	45
FD-PER-9	Perciasacchi	null, 20x + 20y	2	45
FD-PER-13	Perciasacchi	null, 6 + 8	2	45
FD-RUI-1	Ruscia	null, 20x + 20y	2	45
FD-RUI-3	Ruscia	null, 20x + 20y	2	45
FD-RUI-4	Ruscia	null, 20x + 20y	2	45
FD-RUS-1	Russello	2*, 13 + 16/6 + 8	1	45
FD-RUS-3	Russello	2*, 13 + 16/6 + 8	1	45
FD-RUS-4	Russello	null, 13 + 16/6 + 8	1	45
FD-RUS-6	Russello	null, 13 + 16	1	45
FD-SAM-1	Sammartinara	null, 13 + 16	2	47
FD-SCA-1	Scavuzza	null, 6 + 8	2	44
FD-SCO-1	Scorsonera	null, 20	2	45
FD-SCO-2	Scorsonera	null, 20	2	45
FD-TRB-1	Timilia R.B.	null, 6 + 8	2	44
FD-TRB-2	Timilia R.B.	null, 6 + 8	2	44
FD-TRB-4	Timilia R.B.	null, 6 + 8	2	44
FD-TRI-1	Tripolino	null, 6 + 8/13 + 16	1	42
FD-TRN-1	Timilia R.N.	null, 6 + 8	2	44
FD-TRN-3	Timilia R.N.	null, 6 + 8	2	44
FD-TRN-6	Timilia R.N.	null, 6 + 8	2	44
FD-TRN-7	Timilia R.N.	null, 6 + 8	2	44
FD-TRN-8	Timilia R.N.	null, 6 + 8	2	44
FD-TRN-9	Timilia R.N.	null, 6 + 8	2	44
FD-TRN-11	Timilia R.N.	null, 6 + 8	2	44
FD-TRN-12	Timilia R.N.	null, 6 + 8	2	44
FD-URR-1	Urria	null, 20x + 20y	2	47
FD-VAL-2	Vallelunga pubescente	null, 13 + 16	2	45

2.2. Measurement of the Polymeric Glutenin

UPPs are large unextractable polymers, which are related to dough properties. The recent breeding programs led to an improvement in the technological properties of wheat varieties, so it is expected to find a higher %UPP in all modern wheats.

SE-HPLC analysis was performed to quantify the amount and size of glutenin polymer in the grain of Sicilian tetraploid landraces Bidì, Castiglione Glabro, Gioia, Perciasacchi, Russello, Ruscia, Timilia Reste Bianche, Timilia Reste Nere, and two control durum wheat varieties, an old one, Senatore Cappelli, and a modern one, Saragolla.

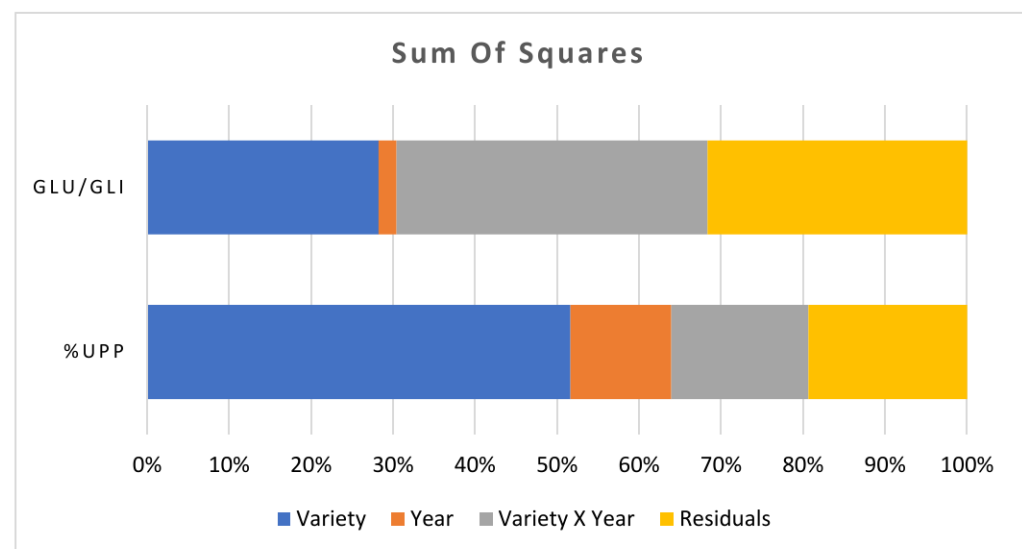
Differences among wheat landraces/varieties and growing seasons were found for %UPP and Glu/Gli ratio (Table 2). In detail, as expected, the modern cultivar Saragolla had a higher %UPP. During 2020, all the genotypes analyzed showed an increase in the content of unextractable polymeric protein compared to the 2019 season except for the old cultivar, Senatore Cappelli, which showed a decrease of 21%, and the conservation varieties Bidì, Castiglione Glabro, Gioia, and Timilia Reste Bianche, in which the %UPP did not change. In both growing seasons, Ruscia showed the lowest content of %UPPs, whereas Russello showed the highest among Sicilian landraces. The %UPP value of the latter one was close to modern variety Saragolla. Instead, Perciasacchi had the largest difference in %UPP (>65%) between the growing seasons of 2019 and 2020.

Table 2. Content of %UPP and Glu/Gli ratio of different landraces related to the two growing seasons.

Name	Type	%UPP		Glu/Gli	
		2019	2020	2019	2020
Bidi	Landrace	33.9 ± 1.0 ab	38.8 ± 2.6 bc	0.480 ± 0.021 c	0.734 ± 0.040 a *
Castiglione Glabro	Landrace	29 ± 1.2 abc	28.1 ± 0.3 de	0.639 ± 0.042 ab *	0.412 ± 0.026 d
Gioia	Landrace	29.4 ± 1.1 abc	31.2 ± 0.8 cde	0.465 ± 0.037 c	0.556 ± 0.004 bcd
Perciasacchi	Landrace	25.9 ± 1.7 c	38.9 ± 3.0 bc *	0.586 ± 0.024 bc	0.505 ± 0.031 cd
Russello	Landrace	36.9 ± 1.2 a	44.2 ± 2.0 ab *	0.622 ± 0.018 ab	0.573 ± 0.029 bc
Ruscia	Landrace	15.1 ± 0.5 d	25.2 ± 1.3 de *	0.560 ± 0.016 bc	0.492 ± 0.056 cd
Saragolla	Modern Cultivar	30.8 ± 3.4 abc	50.7 ± 2.2 a *	0.580 ± 0.031 bc	0.664 ± 0.017 ab *
Senatore Cappelli	Old Cultivar	28.3 ± 1.6 bc *	22.4 ± 1.9 e	0.723 ± 0.017 a *	0.559 ± 0.014 bcd
Timilia Reste Bianche	Landrace	30.1 ± 0.7 abc	30.3 ± 1.0 cde	0.530 ± 0.022 bc *	0.424 ± 0.021 d
Timilia Reste Nere	Landrace	27.2 ± 1.4 bc	33.5 ± 1.1 cd *	0.519 ± 0.031 bc *	0.428 ± 0.018 d

For each landrace, means within columns followed by different lower-case letters refer to the statistical analysis performed using one-way analysis of variance (ANOVA) with post-hoc Tukey HSD test ($p < 0.05$). For each sample, means within row followed by asterisks indicate a significant difference in the two different years. The statistical analysis was performed using Student's *t*-test ($p < 0.05$).

The Glu/Gli ratio was influenced by the growing season differently than the %UPPs (Figure 2). The conservation varieties Bidi and Gioia showed the lowest ratio of Glu/Gli during 2019. Conversely, during the 2020 season, Bidi showed the highest value, even more than the modern variety Saragolla. Castiglione Glabro, Timilia Reste Bianche, and Timilia Reste Nere demonstrated worsening in the Glu/Gli ratio in the 2020 season compared to the previous one. Finally, Saragolla confirmed good technological quality during the season 2020, while Senatore Cappelli was the worst.

**Figure 2.** Percentage of the total sums of squares for the effects of variety, year, interaction between variety and year, and residuals.

The percentages of sums of squares accounting for the effects of the growing season were 12% and 2% for %UPP and Glu/Gli, respectively. The greatest contribution to the difference in Glu/Gli ratio was provided by the interaction of variety x year. Instead, the varietal effect contributed to 52% and 12% for %UPP and Glu/Gli ratio, respectively (Figure 2).

3. Discussion

There has been increasing interest in wheat landraces in recent years, especially in Italy, where tetraploid wheats, particularly durum wheat, have a long tradition of growing and breeding. Sicily is the region that contributes most, with 24 out of the 28 landraces recorded

in the National Register of Conservation Varieties. This germplasm is agronomically and nutritionally interesting and represents a valuable resource to preserve cereal genetic diversity. Products based on wheat landraces, so-called “ancient Sicilian grains”, are present on the shelves of large Italian retailers. However, correspondence with variety is not guaranteed due to a lack of traceability along the supply chain. It is thus important that such landraces are deeply and clearly characterized and traceable.

Within this context, our work aimed to characterize 51 accessions belonging to 22 Sicilian tetraploid wheat landraces and to assess the intra- and inter-accession variability. Since each cultivar has a specific prolamin composition, electrophoretic patterns of both glutenins and gliadins were used as characterization criteria. Several studies have demonstrated that the electrophoretic profile of prolamins is specific for each cultivar and is not influenced by growing conditions, kernel protein content, or incomplete seed maturation [54–56].

Analyses of individual spikes allowed to detect heterogeneity in the same accession, mostly revealed by gliadins pattern. Indeed, 85% of the studied accessions differed by allelic composition in at least one *Gli* locus. This was expected as gliadins are one of the most polymorphic proteins in nature, with 147 alleles in tetraploid wheat identified to date [54]. Cases of intra-varietal non-uniformity of common wheat cultivars have been previously described [56–59]. This variability could be explained as the landraces were obtained by crossing different wheat genotypes that were characterized by different allelic compositions at the *Gli* loci [60] and were not selected for this trait. For this reason, the different ecotypes that constitute a landrace may contribute to its adaptivity, and thereby to the known high plasticity of the local varieties.

The accessions' reference patterns showed different compositions of HMW-GS in the Gioia, Russello, and Perciasacchi landraces. For Gioia, it seems to be a contamination because the different bands were faint and present only in one accession (Figure S1). The Perciasacchi accessions showed the presence of Bx20 + By20 or Bx6 + By8 subunits. The first HMW-GS composition was already described by Visioli et al. [53] in the pure seeds provided by the CREA's seed bank. In this study, the authors analyzed commercial flour labelled as monovarietal and found contaminations regarding the HMW-GS subunits. Most of the samples are characterized by a different profile, with the presence of subunits Bx6 + By8. Our data confirmed that the form showing Bx6 + By8 HMW-GS is the most common in Sicilian Perciasacchi landraces grown by custodian farmers and showed how the institutional seed banks often do not reflect the genetic variation of the actually grown materials.

In the same study [53], the analysis of the flours obtained by Russello showed the HMW-GS composition involving Bx13 + By16 and Bx6 + By8. The presence of Bx6 + By8 was explained by the authors as a contamination with Ruscia. In this paper, the accessions of the same landrace (Russello) showed polymorphism with the presence at the loci *Glu-A1* (null or 2*) and *Glu-B1* (Bx6 + By8 or Bx13 + By16 or both) gluten subunits. In our study, all the accessions of Ruscia highlighted a different composition at *Glu-B1* (Bx20 + By20) compared to those described by Visioli et al. [53] (Bx6 + By8), while the same composition (Bx20 + By20) was observed for Bidì (Margherito). In agreement with our results, another work confirmed the presence of the Bx13 + By16 subunits for Russello, while the same authors described a different HMW-GS (Bx20 + By20 vs. Bx6 + By8 subunits) for Timilia Reste Bianche [35].

The landrace reference patterns showed a different composition in prolamins profiles for all the ancient wheats, confirming that electrophoretic analysis of gliadins and glutenins is a valid method for the traceability of these materials. The only exceptions were for Bidì, Senatore Cappelli, and Capeiti 8, which showed the same prolamin composition. The similarity of gliadins and glutenins profiles is explained by the common origin shared by these varieties. In particular, Bidì and Senatore Cappelli are very similar morphologically, and they were reported to be selected, respectively, by Tunisian landraces Mahmoudi and Jean Retifah; Capeiti 8 was obtained by the cross of Eiti 6 x Senatore Cappelli [5,34,61]. However, De Cillis [62] reported that both Bidì and Senatore Cappelli were selected from

the same Tunisian landrace named Bidì, and, recently, some other studies supported this hypothesis [26,27,63]. To trace these landraces requires a different method, such as SNP genotyping, which was useful for revealing genetic diversity in a large population of two Sicilian landraces, Russello and Timilia [27].

Moreover, in this paper, analysis of storage proteins enabled comparing dough quality among landraces in two different growing seasons. Gluten polymers contribute to the technological quality of the wheat by influencing the rheological properties of the dough, and this can be predicted by the amount of %UPP and Glu/Gli [50,64]. The amount of insoluble polymeric proteins (%UPP) was higher for all the accessions analyzed during the 2020 season due to favorable growing conditions except for Senatore Cappelli, which showed a decrease in these proteins. Furthermore, the low %UPP found in this variety was predictable due to the presence of Bx20 + By20 HMW-GS, correlated with low technological quality [65]. Instead, during the 2019 growing season, Bidì showed a similar %UPP to the modern variety Saragolla and, during the 2020 growing season, a higher Glu/Gli ratio despite having the same HMW composition of Senatore Cappelli. Differences in %UPP between landraces with the same allelic composition were due to differences in storage protein subunit amount and could be explained by differences in gluten strength [35]. Therefore, the 2019 season was characterized by adverse growing conditions, and the Russello variety evidenced possessing a technological quality close to the modern variety Saragolla, although, when the environmental conditions are favorable (as in 2020), the Saragolla values are higher, as expected. Instead, Perciasacchi is the genotype that suffered the most from the adverse weather conditions and had the largest difference in %UPP between the two growing seasons.

4. Materials and Methods

4.1. Plant Material and Experimental Conditions

The collection of Sicilian grains included in this study are 19 *Triticum turgidum* subsp. *durum* (durum wheat), two *Triticum turgidum* subsp. *turgidum*, and one *Triticum turgidum* subsp. *Turanicum* (khorasan wheat) for a total of 51 accessions (Table S1); two historical cultivars (Senatore Cappelli and Trinakria) and three modern durum wheat cultivars (Iride, Core, and Saragolla) were used as controls. The cultivation of these materials was carried out in Sicily in two different locations in 2019 (Palermo, 38°06'54.36" N; 13°21'02.88" E, 46 m a.s.l.) and in 2020 (Vicari, Palermo province, 37°49'29"28 N., 13°34'1"20 E., 640 m a.s.l.) under conventional agricultural regime.

Initially, in gliadin and glutenin gels, the extract from two seeds from each of ten spikes, along with a bulk of seeds obtained from each accession, were run. Subsequently, to compare accessions with each other, gels were prepared using the bulk samples of all accessions from all varieties. Finally, the bulk samples of all accessions from each variety were mixed to obtain a representative bulk of the varieties, which was then used to produce the final gels.

4.2. Extraction of Gluten Protein

Two seeds from each sample spike and five seeds for each sample bulk of the different wheat landraces and commercial varieties were crushed in a mortar with a pestle until a fine powder was obtained. The procedure for extracting gluten protein followed the sequential extraction protocol. The gliadins were extracted from 15 mg of whole flour in 1.5 M dimethyl formamide (1:10, *w/v*) with continuous mixing for 1 h at room temperature, followed by centrifugation at 14,000 × *g* for 10 min. The pellet was stored at −20 °C and subsequently used for the extraction of the glutenin subunits while the supernatant containing the gliadins fraction was collected. The gliadin loading dye (50% saccharose (*w/v*) and 0.02% (*w/v*) dimethyl violet) was added to each sample. The tubes were then vortex-mixed, centrifuged for 2 min at 14,000 × *g*, and 10 µL of the supernatant were used to load the gels.

Extraction of glutenin fraction was conducted as described by Ibba et al. [66] with minor modifications. Briefly, the pellet obtained from the first dimethyl formamide treatment was washed two times with 750 µL of 50% 1-propanol and, after centrifugation, the supernatant was discarded to remove any remaining gliadins from the pellet. A volume of 100 µL of a solution with dithiothreitol (DTT) at 1.5% (*w/v*) formed with 50 µL of 1-propanol at 50% (*v/v*) and 50 µL of Tris-HCl 0.08 M pH 8.0 was then added to the pellet. The tubes were mixed in a vortex and incubated for 30 min at 65 °C for the extraction of glutenin fraction. After the centrifugation at 10,000× *g* for 2 min, 100 µL of a solution with vinylpyridine at 1.4% (*v/v*) formed with 50 µL of propanol at 50% (*v/v*) and 50 µL Tris-HCl 0.08 M pH 8.0 was added to the tubes, which were then mixed with a vortex, incubated for 15 min at 65 °C, and centrifuged for 2 min at 14,000× *g* for alkylation of glutenin subunits. The supernatant containing the glutenin fraction was recovered and transferred to new tubes containing the loading solution 2× (Tris-HCl pH 6.8, 2% SDS, 40% glycerol and 0.02% bromophenol). The samples were mixed in a vortex, incubated for 5 min at 90 °C, and then centrifuged for 2 min at 14,000× *g*, and 10 µL of the supernatant were loaded on polyacrylamide gels.

4.3. Electrophoretic Separation

A-PAGE was performed using polyacrylamide gels (8 × 13 cm) with 8% of T value (total acrylamide concentration) and 1.25% of C value (cross-linker concentration) containing 40 mM aluminum lactate, 260 mM lactic acid, and 0.002 g of ascorbic acid. After electrophoretic separation at 25 mA, the gels were stained with a Coomassie R-250 solution fixed in 5% ethanol and 12% trichloroacetic and de-stained in deionized water.

SDS-PAGE was performed using separating gel with 15% T value and 1.3% of C value. The main gel was prepared using 0.36 M Tris-HCl, pH 8.8, and 0.1% SDS. Gels were run at 12.5 mA per gel for 20 h and stained with brilliant blue G-colloidal solution fixed in 20% methanol and 10% phosphoric acid and de-stained in deionized water.

The two biotypes of the Italian durum wheat cultivar Lira 42 and Lira 45 were used as standards of γ-gliadins γ-42 and γ-45, as well as of LMW-1 and LMW-2 types. As HMW-GS standards, the Italian durum wheat cultivars Core (7 + 8), Iride (7 + 8), Saragolla (6 + 8), and Senatore Cappelli (20x + 20y) were used.

4.4. Analysis of Unextractable Polymeric Proteins (%UPP) by SE-HPLC

SE-HPLC was used to measure the percentage of non-extractable polymer proteins (%UPP). The extraction of the SDS-soluble fraction and the SDS-insoluble fraction was performed as reported by Gagliardi et al. [67], except for the sonication, which was performed with the probe-type sonicator SONICS Vibracell model VC 50T (power 50 W, frequency 20 KHz) for 15 s. Briefly, soluble fraction was first extracted from semolina using SDS-phosphate buffer. The remaining pellet was resuspended in the same buffer and sonicated to obtain the UPP portion of semolina protein. The HPLC equipment used was a HPLC Knauer Smartline system equipped with two Smartline 1000 pumps, one HT300L autosampler, one Smartline 2600 UV detector, and a TSKgel SuperSW3000 column (300 mm × 4.6 mm). The flow rate was 0.35 mL/min, and the detection was at 214 nm. Every sample was analyzed in two replicates. The area under the first peak of the soluble fraction was called P1_s, while the one under the other peaks was named P2_s. The areas of the insoluble fraction were named, respectively, P1_{ns} and P2_{ns} (Figure S6). The %UPP and the Glu/Gli ratio have been determined as

$$\%UPP = \left(\frac{P1_{ns}}{P1_{ns} + P1_s} \right) \times 100$$

$$\frac{Glu}{Gli} = \frac{P1_{ns} + P1_s + P2_{ns}}{P1_s}$$

4.5. Statistical Analysis

The %UPP and Glu/Gli values of tetraploid varieties cultivated in the same year were analyzed by one-way analysis of variance (ANOVA), and pairwise analysis was carried out using the Tukey Honestly Significant Difference test (Tukey test) ($p < 0.05$). The comparison of these values between the two years was analyzed with Student's *t*-test ($p < 0.05$). For the calculation of the Sum of Squares, a two-way ANOVA was performed.

All statistical processing was completed with R, version 4.1.3 [68].

5. Conclusions

Ancient Sicilian wheat holds significant importance in producing traditional niche products that fuel the local economy. Despite a growing demand for tetraploid cereal products in the traditional retail market, limited production raises concerns about potential fraud. The current lack of traceability throughout the supply chain poses a challenge to ensure alignment between certified seeds and final products. Semolina labeled as monovarietal or its derived products may not accurately represent the declared variety due to this traceability gap. Additionally, the conservation of these wheat varieties relies on custodian farmers, and these populations often exhibit a level of heterogeneity.

Our study indicates that the biochemical profile of prolamins effectively distinguishes among ancient tetraploid wheat varieties, addressing traceability issues to the mutual benefit of consumers and producers. Furthermore, the comparison among accessions by custodian farmers revealed intra- and inter-accession heterogeneity for prolamins profiles. In fact, these landraces have been naturally selected for agronomic traits and not for gluten quality. Particularly, the polymorphic nature of gliadin enables more distinct discrimination among different accessions.

In conclusion, the biochemical profile of prolamins represents a valid tool to trace ancient wheats, contributing to the preservation of biodiversity and avoiding fraud along the supply chain.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/plants13050741/s1>, Figures S1–S5: Electrophoresis separation of gluten proteins; Figure S6: examples of SE-HPLC chromatograms used for %UPP calculation; Table S1: List of wheat samples characterized by gluten protein; Table S2: High-molecular-weight (Glu-HMW), low-molecular-weight (Glu-LMW), and gliadins (Gli) patterns of Sicilian wheat landraces. The protein profile is defined as heterogeneous when at least one sample analyzed has a different pattern.

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Following the initial assessment of variability among Sicilian tetraploid wheat conservation varieties, we selected the five most representative (Bidi, Perciasacchi, Russello, Timilia reste bianche, and Timilia reste nere) for further characterization.

In the years, it has been claimed that the consumption of old wheat could be better for people suffering of NCWS and IBS. Among the compound suspected to cause these pathologies, there are ATIs and fructans. To assess potential differences in the accumulation of these compounds between conservation varieties and modern cultivars, a multi-environment trial was conducted over three consecutive years at two distinct locations in Sicily.

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5 Chapter III - ATI, fructans and predictive assessment of technological quality of Sicilian tetraploid conservation and modern wheat varieties

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ATI, fructans and predictive assessment of technological quality of Sicilian tetraploid conservation and modern wheat varieties

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ABSTRACT

Recently, there has been an increasing interest in wheat landraces and historical varieties mainly cultivated at the beginning of the 19th century, defined as conservation varieties. Most of the Italian tetraploid conservation varieties originate from Sicily, where they are yet boosting the local economy. One of the main reasons for the growing interest in these varieties is the consumers' perception that they are healthier and safer than their modern counterparts. It is thought that their consumption could ameliorate symptoms of non-celiac wheat sensitivity (NCWS) and irritable bowel syndrome (IBS). Amylase/trypsin-inhibitors (ATIs) and fructans are among the wheat grain components linked to these conditions. In this study we quantified ATI and fructans in five Sicilian conservation varieties (Bidi, Perciasacchi, Russello, Timilia reste bianche, Timilia reste nere) and three modern cultivars (Iride, Orizzonte, Simeto) in a multi-environment trial. Moreover, we calculated the percentage of unextractable polymetric proteins (%UPP) to predict their technological quality. Our findings revealed no significant differences between conservation varieties and modern cultivars for total ATI content, although variations were observed in the composition of individual ATIs. The fructan content was lower in conservation varieties, especially in Bidi and Russello. Overall, %UPP was generally higher in modern cultivars, although Bidi often displayed comparable values. The environmental influence was significant for all analysed traits; however, high heritability was yet observed for all, except for total ATI content. Our results indicate that only minor differences in grain composition exist between tetraploid conservation varieties and modern cultivars.

1. Introduction

Wheat is one of the most cultivated crops in the world and it is the major staple food in the human diet. Among cereals, it is the second for production quantity and the first for harvested area, thanks to its good adaptability to different climatic regions (FAO, 2022).

Wheat is the term that indicates different species belonging to the *Triticum* genus. The most important species is hexaploid common wheat (*Triticum aestivum* L., genome AABBDD, $2n = 6x = 42$), representing 95 % of wheat cultivated worldwide (Shewry, 2009). The remaining 5 % is mainly represented by tetraploid durum wheat (*Triticum turgidum* L. ssp. *durum* (Desf.) Husn., genome AABB, $2n = 4x = 28$), mostly cultivated in the Mediterranean area and in Canada (Xynias et al., 2020).

Wheat consumption is associated to different adverse reactions, such as celiac disease (CD), baker's asthma, IgE-mediated food allergy, wheat-dependent exercise-induced anaphylaxis (WDEIA), non-celiac wheat sensitivity (NCWS) and irritable bowel syndrome (IBS) (Brouns et al., 2019). Recently, there has been a renewed interest in the old landraces and historical varieties cultivated before the advent of the Green Revolution. These are often referred to as "ancient grains", but the term "heritage wheat" is more appropriate to distinguish them from wheat relatives including einkorn (*T. monococcum*, genome AA, $2n = 2x = 14$) or emmer (*T. dicoccoides*, genome AABB, $2n = 4x = 28$) or other subspecies of *T. turgidum*, such as Khorasan wheat (*T. turgidum* ssp. *turanicum*) (Brouns et al., 2022).

In Italy, this renewed interest is particularly notable in Sicily, a

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region with a long history of cultivation of durum wheat and particularly rich in landraces (Fiore et al., 2022; Spina et al., 2023). Landraces and historical varieties “which are naturally adapted to local and regional conditions and threatened by genetic erosion” can be inscribed in the Italian Nation Catalogue of agricultural species as conservation varieties. Nowadays, 24 of the 28 durum wheat conservation varieties present in the catalogue are Sicilian. In this region, these varieties boost the local economy thanks to the establishment of a micro supply chain.

Ancient grains and heritage wheat are perceived by the consumers as safer and healthier than modern wheat, in some cases in absence of scientific evidence. In fact, while there is a common belief that affirms that breeding led to an increase of the toxicity of wheat gluten, many studies found that the content of gluten, gliadin and CD-active epitopes has not changed over time, and in some “ancient grains” it is even higher than in modern cultivars (Brouns et al., 2022; Prandi et al., 2017). There are some preliminary studies that suggest that the consumption of ancient grains could be more tolerable by NCWS and IBS patients. Sofi et al. (2014) reported an improvement in IBS symptoms and inflammatory profile after consumption of Khorasan wheat in comparison to modern wheat. Alvisi et al. (2017) found that proteins of one modern wheat variety stimulate more proinflammatory cytokine secretion by peripheral blood mononuclear cells in NCWS pediatric patients than proteins of two varieties of ancient grains (KAMUT® Khorasan wheat and Italian historical variety Cappelli). Ianiro et al. (2019) conducted a double-blind randomized cross-over trial of 42 patients and found that consumption of pasta made with organic Cappelli caused less symptoms than standard pasta consumption in NCWS patients. Seidita et al. (2022) monitored “ancient grain” consumption in 233 NCWS patients and found an association with a delayed diagnosis. Additionally, their findings indicated that adherence to a wheat-free diet led to symptom improvement. In this latter study, the most consumed “ancient grains” were Timilia and Russello, two Sicilian landraces, Perciasacchi and Kamut®, two Khorasan wheat varieties, and Cappelli. However, all these studies lack an adequate comparison with modern varieties. To thoroughly assess the potential for similar effects, the range of modern varieties examined should be expanded, considering their diverse genetic backgrounds.

NCWS is characterized by intestinal (diarrhea, abdominal pain, bloating) and extra-intestinal (dermatitis, fatigue, headaches) symptoms and there is strong evidence that it is caused by the activation of innate immunity in response of wheat proteins (Schuppan et al., 2015). Noteworthy, it has been demonstrated that the ingestion of amylase/trypsin-inhibitors (ATI) induces a potent innate immune response and the activation of myeloid cells via the interaction with the TLR4-MD2-CD14 complex (Junker et al., 2012; Zevallos et al., 2017).

ATI are water-soluble (non-gluten) proteins accounting for about 4 % of the kernel proteins and their role is to protect the kernel from insect and mammalian amylase (Dupont et al., 2011). They share a high sequence homology and similar secondary structure characterized by four α -helices and four to five disulphide bridges (Carbonero and García-Olmedo, 1999; Oda et al., 1997). ATI are encoded by at least 19 genes and can be divided in four groups: i) wheat monomeric amylase-inhibitor WMAI (0.28), ii) wheat homodimeric amylase-inhibitors WDAI (0.19, 0.53), iii) wheat heterotetrameric amylase-inhibitors WTAI (CM1, CM2, CM3, CM16, CM17) and iv) monomeric trypsin-inhibitors (CMX1/3, CMX2) (Geisslitz et al., 2021). Other protease inhibitors that share sequence similarities with ATI can be found in wheat, such wheat trypsin-inhibitor (WTI), wheat chymotrypsin-inhibitor (WCI) and wheat endogenous amylase/subtilisin-inhibitor (WASI) (Altenbach et al., 2011). It has been reported that ATI levels increase with ploidy, with einkorn showing a content close to zero (Geisslitz et al., 2020). Two studies on the ATI content of various common wheat accessions released between 1891 and 2013 revealed that breeding has not altered their levels (EL Hassouni et al., 2021; Geisslitz et al., 2023).

Another class of compounds suspected to cause NCWS are

fermentable oligo-, di-, and monosaccharides and polyols (FODMAPs) (Biesiekierski et al., 2013; Dieterich et al., 2019). The major FODMAPs present in wheat grains are fructans, polymers composed of fructose molecules. In particular, wheat contains a mix of branched fructans of the graminan-type and neotype, both characterized by $\beta(2-1)$ and $\beta(2-6)$ fructosyl linkages (Verspreet et al., 2015). Durum wheat and emmer are reported to contain less fructans than common wheat and einkorn, although the main differences are given by the genetic differences between varieties (Ribeiro et al., 2022; Ziegler et al., 2016).

Despite the claims regarding the health benefit of the consumption of conservation varieties, there is poor knowledge about the composition of their grains. In this study, we performed a multi-environment trial with five Sicilian conservation varieties (Bidi, Perciasacchi, Russello, Timilia reste bianche and Timilia reste nere) and three modern cultivars (Iride, Orizzonte and Simeto) and quantified their ATI and fructan content. Moreover, we calculated their percentage of unextractable polymeric protein (%UPP) for technological quality prediction.

The aim of this work was to determine whether there are differences in kernel composition between conservation varieties and modern varieties, as well as to assess the influence of the environment on ATI and fructan accumulation.

2. Results

2.1. Quantification of ATIs in conservation and modern durum wheat varieties

Thirteen single ATIs were quantified in five conservation varieties and three modern cultivars grown at two Sicilian locations for three years (2021-2022-2023) using a targeted liquid chromatography-tandem mass spectrometry (LC-MS/MS) method. Means and standard errors of biological replicates are reported in [Supplementary Table S1](#).

The WCI content was below the limit of detection (LOD) in each sample of all the varieties analysed. The content of CM1 and CM17 was below the LOD for most samples, except in few cases ([Supplementary Fig. S1](#)). However, given the very low values observed (<50 $\mu\text{g/g}$ for CM1 and <15 $\mu\text{g/g}$ for CM17), these could potentially be attributed to contamination or false positive identification. For this reason, WCI, CM1 and CM17 were not included in the subsequent analysis.

The content of individual ATIs in each variety and “type” is reported in [Figs. 1 and 2](#), respectively. The ATI composition of each variety is reported in [Supplementary Fig. S2](#).

The tetrameric ATIs CM3, CM2 and CM16 were the most abundant in the durum wheat varieties analysed, accounting for 70 %–80 % of the total ([Supplementary Fig. S2](#)).

In general, no significant differences were observed between conservation varieties and modern cultivars for CM3, the most abundant ATI (p-value = 0.671) ([Fig. 2a](#)). Its content was higher in modern varieties cultivated in Caltavuturo (year 2021 p-value = 0.0001 and year 2022 p-value = 0.0001), while it was higher in conservation varieties cultivated in Caltavuturo (year 2023 p-value < 0.0001) and Selinunte (year 2023 p-value = 0.0013) ([Supplementary Fig. S3](#)). We found high CM3 content variability in individual varieties across the different environments, with no discernible trend ([Supplementary Fig. S4](#)).

Conservation varieties had the significantly highest content of CM2 and CM16 (p-value = 0.0037 and p-value = 0.0002 respectively) ([Fig. 2b–c](#)). For CM2, there was variation across the environments, since there was no significant difference in growing season 2021 in Caltavuturo (p-value = 0.696) and Selinunte (p-value = 0.402). Differently modern varieties had a significantly higher content in Caltavuturo (year 2022 p-value = 0.010) ([Supplementary Fig. S5](#)). In the case of CM16, no significant differences were found in Caltavuturo (2021) (p-value = 0.188) and 2022 (p-value = 0.735) and in Selinunte (2021) (p-value = 0.355) ([Supplementary Fig. S6](#)). Orizzonte had the significantly lowest content of CM2 and CM16 ([Fig. 1b–c](#)), although this was not consistent across the environments ([Supplementary Fig. S7](#) and [Supplementary](#)

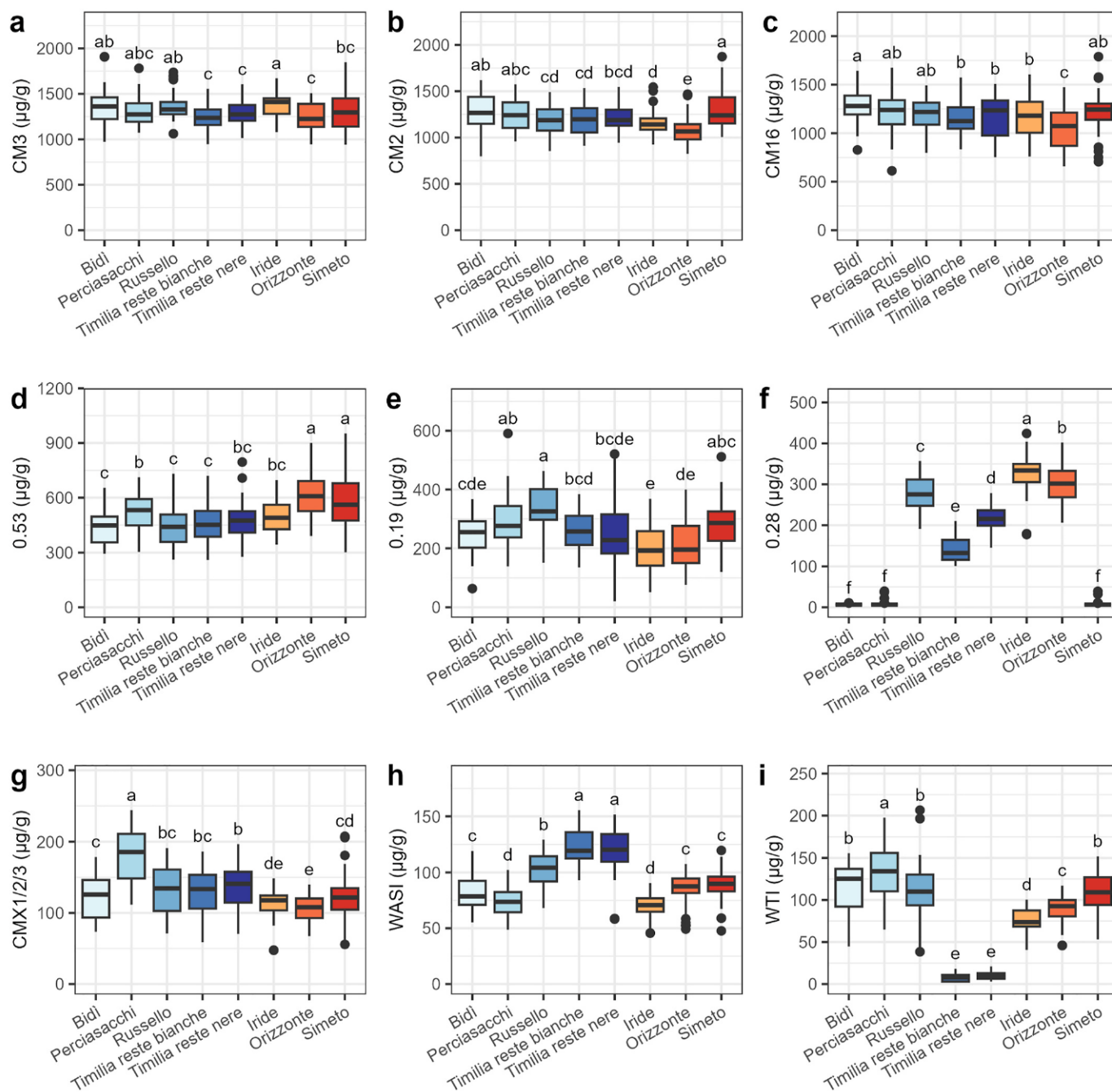


Fig. 1. – Boxplot of the content of CM3 (a), CM2 (b), CM16 (c), 0.53 (d), 0.19 (e), 0.28 (f), CMX 1/2/3 (g), WASI (h), WTI (i), averaged across three years and two locations. The box represents the interquartile range (IQR), with the line inside indicating the median. The whiskers extend to the data range within $1.5 \times \text{IQR}$ from the first and third quartiles, while dots represent outliers. Each boxplot represents 12 biological replicates ($n = 12$). All technical replicates have been plotted. Means not sharing any letter are significantly different (multiple comparison with Šidák correction, $\alpha = 0.05$). Conservation varieties are represented by shades of blue, while modern varieties are represented by shades of red. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Fig. S8).

Dimeric ATIs 0.53 and 0.19 accounted for approximately 8–13 % and 4–6 % of the total, respectively (Supplementary Fig. S2). The content of 0.53 was significantly higher in modern varieties (t -test, p -value < 0.0001) (Fig. 2d), with this trend consistently observed across all the environments (Supplementary Fig. S9). On the contrary, the content of 0.19 was significantly more abundant in conservation varieties (t -test, p -value < 0.0001) (Fig. 2e), but in Caltavuturo (2021) (p -value = 0.080) and 2022 (p -value = 0.128) and Selinunte 2022 (p -value = 0.078) no significant differences were found (Supplementary Fig. S10). Among

modern varieties, Orizzonte and Simeto tended to have the significantly highest content of 0.53 (Fig. 1d), with discrepancies across environments (Supplementary Fig. S11). High variability between individual varieties was found for 0.19 across environments (Fig. 1e and Supplementary Fig. S12).

Monomeric ATI 0.28 comprised about 3–7 % of the total ATI in Russello, Timilia reste blanche, Timilia reste nere, Orizzonte and Irìde, but was absent in Bidì, Perciasacchi and Simeto (Fig. 1f and Fig. S2). For this reason, these three varieties were excluded from the “Type x Environment” model. Within this model, modern cultivars exhibited a

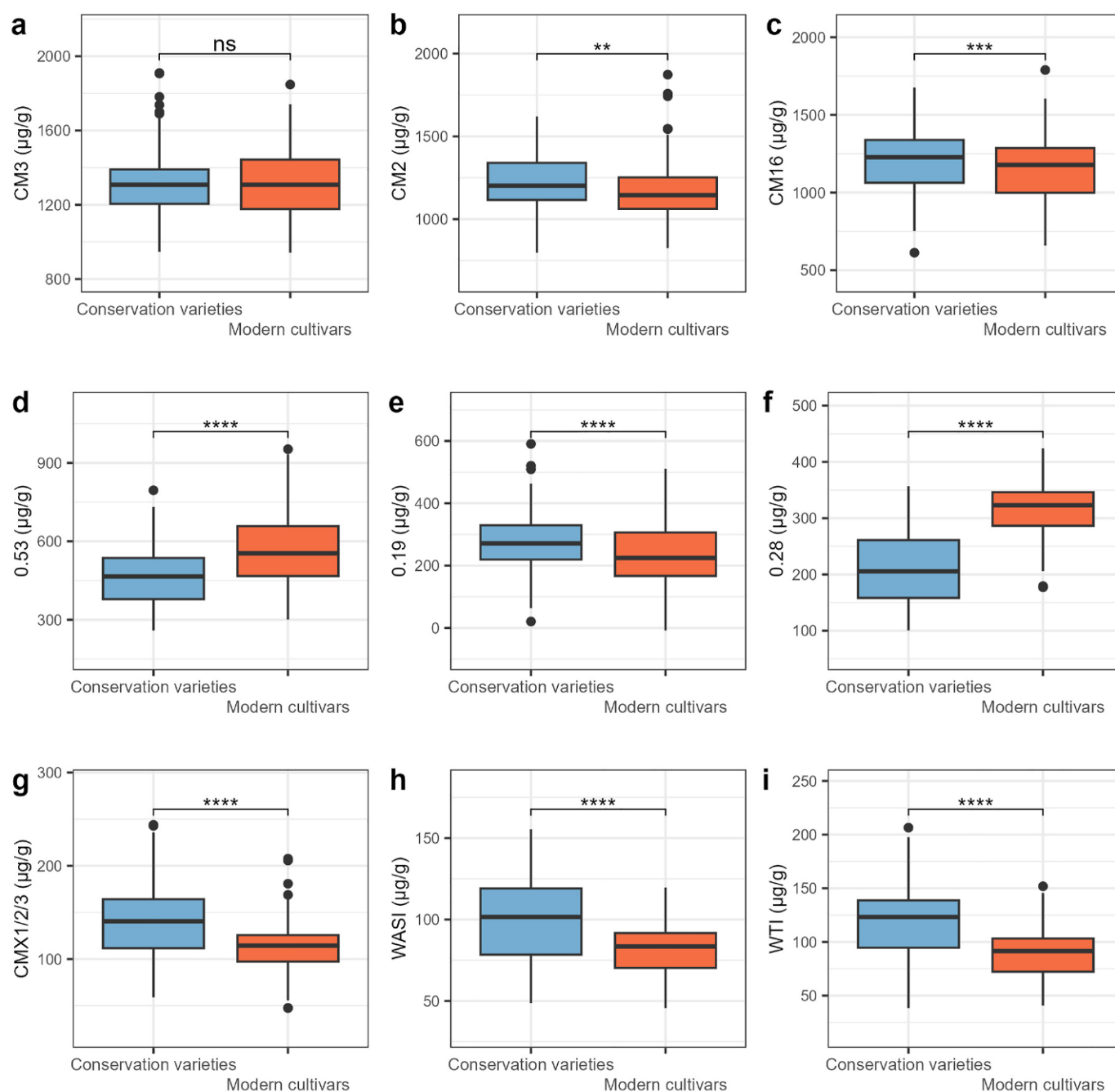


Fig. 2. – Boxplot of the content of CM3 (a), CM2 (b), CM16 (c), 0.53 (d), 0.19 (e), 0.28 (f), CMX1/2/3 (g), WASI (h), WTI (i) of conservation varieties ($n = 60$) and modern cultivars ($n = 36$), averaged across three years and two locations. It is necessary to underline that, in case of panel (f) only varieties in which 0.28 was expressed have been included, whereas in case of panel (i), Timilia reste bianche and Timilia reste nere have not been included because the content of WTI is significantly lower than in the other conservation varieties. The box represents the interquartile range (IQR), with the line inside indicating the median. The whiskers extend to the data range within $1.5 \times$ IQR from the first and third quartiles, while dots represent outliers. All technical replicates have been plotted. T-test on estimated marginal means has been used to evaluate differences (ns = p -value > 0.05 ; * = p -value < 0.05 ; ** = p -value < 0.01 ; *** = p -value < 0.001 , **** = p -value < 0.0001).

significantly higher content of ATI 0.28 (t -test, p -value < 0.0001) in all the environments (Fig. 2f and Supplementary Fig. S13). Among the varieties containing ATI 0.28, Timilia reste bianche and Timilia reste nere showed the lowest content, with the difference being significant across all environments only for Timilia reste bianche (Supplementary Fig. S14).

The content of CMX1/2/3 was significantly higher in conservation varieties (p -value < 0.0001) (Fig. 2g), a trend that remained consistent across all environments except for Caltavuturo 2021 (p -value = 0.768) (Supplementary Fig. S15). Perciasacchi showed the significantly highest

content of CMX1/2/3 in all the environments (Supplementary Fig. S16).

Further, the content of WASI was significantly higher in conservation varieties (p -value < 0.0001) (Fig. 2h), although no significant differences were detected in Caltavuturo (2022) (p -value = 0.101) and Selinunte 2021 (p -value = 0.821) (Supplementary Fig. S17). The highest WASI content was found in Timilia reste bianche and Timilia reste nere (Fig. 1h and Supplementary Fig. S18). Timilia reste bianche and Timilia reste nere had the significantly lowest content of WTI, close to the LOD (respectively 7.74 ± 0.71 $\mu\text{g/g}$ and 9.90 ± 0.90 $\mu\text{g/g}$) (Fig. 1i and Supplementary Fig. S19). These two varieties were excluded from the

“Type x Environment” model. WTI content was significantly higher in conservation varieties (p-value<0.0001) (Fig. 2i). Significant differences were observed in all environments except for Caltavuturo 2022 (p-value = 0.178) and Selinunte 2021 (p-value = 0.160) (Supplementary Fig. S20).

Finally, no significant differences were found in total ATI content between conservation varieties and modern cultivars (p-value = 0.960) (Fig. 3a). Throughout environments, contrasting results were found: conservation varieties had the lower content in Caltavuturo (2021) (p-value<0.0001) and 2022 (p-value = 0.0002) but the higher content in Caltavuturo (2023) (p-value<0.0001) and Selinunte 2023 (p-value = 0.007). In the other two environments, no significant differences were found (Selinunte, 2021, p-value = 0.830; Selinunte, 2022, p-value = 0.098) (Supplementary Fig. S21). When looking at individual varieties, there were small differences (Fig. 3b). Timilia reste bianche had the lowest total ATI content (4.74 ± 0.07 µg/g), significantly lower than Perciasacchi, Russello, Irde and Simeto. Instead, Russello showed the highest total ATI content (5.18 ± 0.08 µg/g), significantly higher than Bidì, Timilia reste bianche, Timilia reste nere and Orizzonte. Nonetheless, data across environments showed a huge variability, with no clear patterns emerging (Supplementary Fig. S22).

The Student's t-test ($\alpha = 0.05$) was performed to compare the total ATI content of varieties containing ATI 0.28 (Russello, Timilia reste bianche, Timilia reste nere, Irde, Orizzonte) and those that do not (Bidì, Perciasacchi, Simeto). No significant differences were found (p-value = 0.341) (Supplementary Fig. S23).

2.2. Environmental influence on ATI accumulation

Two different linear mixed effect models were fitted: one including the effect of the factors “variety”, “environment” and their interaction (“Variety x Environment” model); one including the effect of the factors “breeding type” (conservation variety or modern cultivar, from now on referred to as “type”), “environment” and their interaction (“Type x

Environment” model). Timilia reste bianche and Timilia reste nere were excluded from the “Type x Environment” model of WTI, due to its low content. Two-way ANOVA was performed on both the models and the results are reported in Tables 1 and 2.

The main effects “variety” and “environment”, as well as their interaction were significant for all the ATIs and for the total ATI content (Table 1), indicating that their accumulation is influenced both by genotypic and environmental factors.

Slightly different results emerged from the ANOVA on the “Type x Environment” model. No significant effect of the interaction was observed for ATI 0.53 ($F_{5,262.21} = 2.032$, p-value = 0.075) and ATI 0.28 ($F_{5,262.21} = 1.733$, p-value = 0.130) (Table 2). In both cases, the effect of “type” and “environment” factors were significant. Moreover, no significant effect of the “type” was found for ATI CM3 ($F_{5,262.21} = 0.180$, p-value = 0.671) and total ATI ($F_{5,262.21} = 0.002$, p-value = 0.960) (Table 2).

2.3. Correlation analysis of different ATIs

Pearson correlation analysis was conducted to assess relationships between individual ATIs, with results presented in Fig. 4.

The strongest positive correlation was observed between CM2 and CM3 ($r = 0.73$, p-value<0.05). Weak positive correlations were also reported for CM2 and CM16 ($r = 0.46$, p-value<0.05), CM3 and CMX1/2/3 ($r = 0.48$, p-value<0.05), CM3 and WTI ($r = 0.41$, p-value<0.05), CM2 and CMX1/2/3 ($r = 0.40$, p-value<0.05). A weak negative correlation was found between WASI and WTI ($r = -0.51$, p-value<0.05). Other significant correlations were found, but they are not discussed further due to their low Pearson coefficients ($r < 0.4$).

2.4. Genotypic and environmental effects on fructan accumulation in conservation and modern durum wheat varieties

The results regarding fructan were reported as g/100g

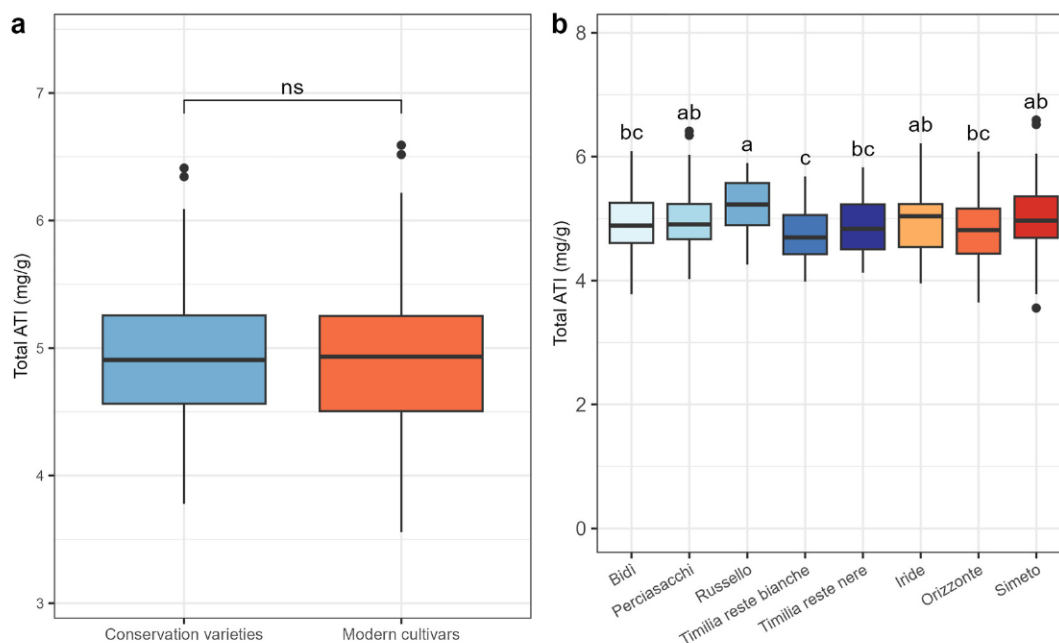


Fig. 3. – Boxplot of the total ATI content between a) types and b) varieties, averaged across three years and two locations. The box represents the interquartile range (IQR), with the line inside indicating the median. The whiskers extend to the data range within $1.5 \times$ IQR from the first and third quartiles, while dots represent outliers. All technical replicates have been plotted. In a) T-test on estimated marginal means has been used to evaluate differences between conservation varieties ($n = 60$) and modern cultivars ($n = 36$) (ns = p-value>0.05; * = p-value<0.05; ** = p-value<0.01, *** = p-value<0.001, **** = p-value<0.0001). In b) each boxplot represents 12 biological replicates ($n = 12$), means not sharing any letter are significantly different (Multiple comparison with Sidák correction, $\alpha = 0.05$). Conservation varieties are represented by shades of blue, while modern cultivars are represented by shades of red. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 1
– ANOVA results for the “Variety x Environment” model. For each factor F statistics, p-value and significance are reported. ns = p-value>0.05; * = p-value<0.05; ** = p-value<0.01; *** = p-value<0.001.

Variety				Environment			Variety x Environment		
Variable	F statistic	p-value	Sig	F statistic	p-value	Sig	F statistic	p-value	Sig
CM3	11.825	8.08E-13	***	28.891	2.70E-06	***	7.052	4.40E-21	***
CM2	18.990	7.24E-20	***	38.843	5.30E-07	***	5.007	2.82E-14	***
CM16	10.512	1.99E-11	***	53.870	8.42E-08	***	2.233	2.40E-04	***
0.53	27.935	1.56E-27	***	86.963	5.51E-09	***	1.989	1.54E-03	**
0.19	12.348	2.29E-13	***	6.831	3.12E-03	**	2.083	7.64E-04	***
0.28	889.328	8.59E-161	***	4.883	1.14E-02	*	3.317	3.33E-08	***
CMX1/2/3	98.533	4.31E-65	***	46.890	1.84E-07	***	5.694	1.22E-16	***
WASI	149.958	2.45E-81	***	55.032	7.57E-08	***	5.391	1.31E-15	***
WTI	499.712	9.70E-134	***	22.079	1.14E-05	***	11.202	7.38E-33	***
Total ATI	8.378	4.24E-09	***	32.307	1.47E-06	***	7.399	3.61E-22	***
Fructans	18.985	7.21E-20	***	57.850	5.74E-08	***	6.724	4.78E-20	***
UPP	43.490	1.40E-38	***	39.099	5.12E-07	***	6.750	4.02E-20	***

Table 2
– ANOVA results for the “Type x Environment” model. For each factor F statistics, p-value and significance are reported. ns = p-value>0.05; * = p-value<0.05; ** = p-value<0.01; *** = p-value<0.001.

Type				Environment			Type x Environment		
Variable	F statistic	p-value	Sig	F statistic	p-value	Sig	F statistic	p-value	Sig
CM3	0.180	6.71E-01	ns	28.016	1.86E-06	***	13.479	1.05E-11	***
CM2	8.596	3.67E-03	**	36.938	3.12E-07	***	8.325	2.55E-07	***
CM16	14.021	2.22E-04	***	55.342	2.85E-08	***	3.568	3.87E-03	**
0.53	89.747	1.67E-18	***	64.129	5.91E-09	***	2.032	7.46E-02	ns
0.19	19.306	1.62E-05	***	7.331	1.60E-03	**	3.593	3.68E-03	**
0.28	135.259	7.50E-23	***	3.912	2.22E-02	*	1.733	1.30E-01	ns
CMX1/2/3	82.651	2.49E-17	***	39.795	1.67E-07	***	5.402	9.52E-05	***
WASI	62.882	6.35E-14	***	14.881	4.29E-05	***	4.170	1.16E-03	**
WTI	95.684	1.46E-18	***	23.183	8.86E-06	***	11.897	5.01E-10	***
Total ATI	0.002	9.60E-01	ns	34.552	5.65E-07	***	12.574	5.92E-11	***
Fructans	21.155	6.60E-06	***	35.072	2.71E-07	***	3.966	1.75E-03	**
UPP	105.747	4.65E-21	***	44.937	6.22E-08	***	7.290	2.05E-06	***

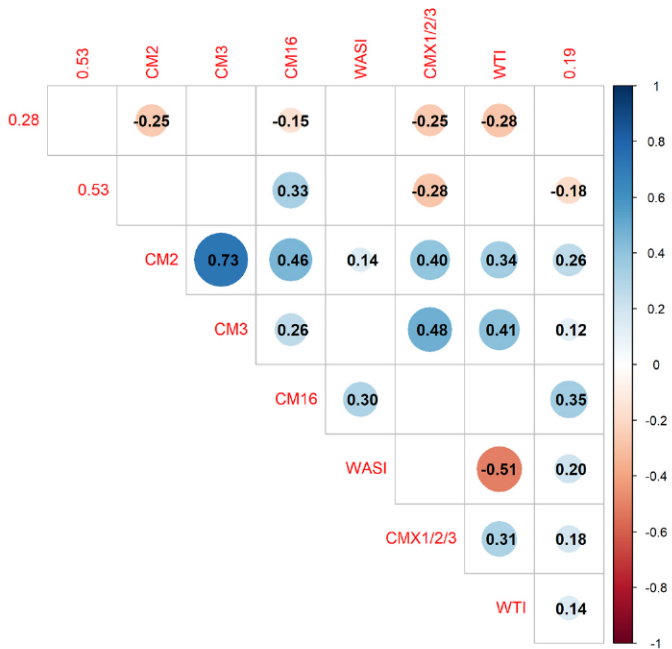


Fig. 4. – Graph showing the Pearson correlation coefficient (r) between individual ATI. Only significant correlations are shown (p-value<0.05).

(Supplementary Table S1) and analysed using two different linear mixed models: “Variety x Environment” and “Type x Environment”. ANOVA results for both models are reported in Tables 1 and 2 A significant effect

of both main factors, as well as their interaction, was observed, highlighting the influence of both genotype and environment effects on fructan accumulation.

Modern varieties showed generally a significantly higher fructan content than conservation varieties (p-value<0.0001) (Fig. 5a). However, the difference was not significant in Caltavuturo (2021) (p-value = 0.890), Selinunte 2022 (p-value = 0.395) and 2023 (p-value = 0.150) (Supplementary Fig. S24).

Bidi and Russello showed the significantly lowest fructan content, 1.29 ± 0.04 g/100g and 1.24 ± 0.03 g/100g, respectively (Fig. 5b). There were no differences among the other varieties, which had fructan levels ranging from 1.50 g/100g to 1.62 g/100g on average. Across different environments, Bidi and Russello always had the lowest fructan content but, in all cases their levels were statistically comparable to those of other varieties (Supplementary Fig. S25).

2.5. Effect of variety and environment on unextractable polymeric protein (%UPP)

The percentage of UPP was calculated using size-exclusion chromatography, comparing the chromatogram of the polymeric protein extractable in a solution containing SDS before and after sonication. As before, two linear mixed effect models were fitted: “Variety x Environment” and “Type x Environment”. ANOVA results indicate that in both models the main effects and their interaction are significant (Tables 1 and 2).

Modern varieties showed a significantly higher %UPP than conservation varieties (p-value<0.0001) (Fig. 6a). The difference was significant in all the environments except for Caltavuturo 2021 (p-value = 0.925) (Supplementary Fig. S26).

The three modern varieties, Iride, Orizzonte and Simeto, had a %UPP

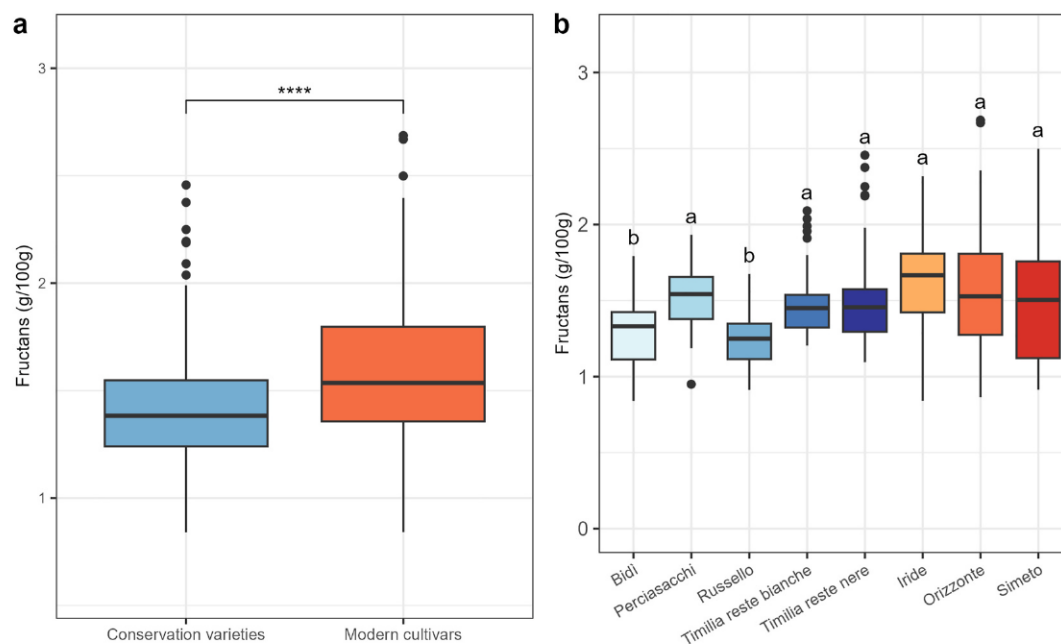


Fig. 5. – Boxplot of the fructans content between a) types and b) varieties, averaged across three years and two locations. The box represents the interquartile range (IQR), with the line inside indicating the median. The whiskers extend to the data range within $1.5 \times$ IQR from the first and third quartiles, while dots represent outliers. All technical replicates have been plotted. In a) T-test on estimated marginal means has been used to evaluate differences between conservation varieties ($n = 60$) and modern cultivars ($n = 36$) (ns = p -value > 0.05 ; * = p -value < 0.05 ; ** = p -value < 0.01 , *** = p -value < 0.001 , **** = p -value < 0.0001). In b) each boxplot represents 12 biological replicates ($n = 12$), means not sharing any letter are significantly different (Multiple comparison with Šidák correction, $\alpha = 0.05$). Conservation varieties are represented by shades of blue, while modern cultivars are represented by shades of red. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

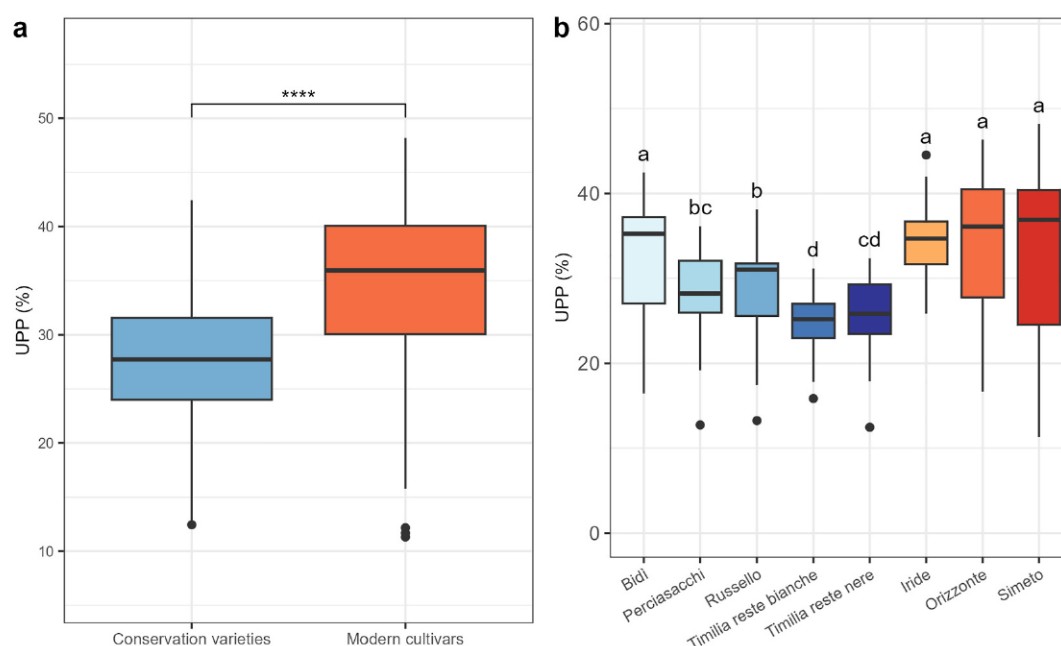


Fig. 6. – Boxplot of the %UPP between a) types and b) varieties, averaged across three years and two locations. The box represents the interquartile range (IQR), with the line inside indicating the median. The whiskers extend to the data range within $1.5 \times$ IQR from the first and third quartiles, while dots represent outliers. All technical replicates have been plotted. In a) T-test on estimated marginal means has been used to evaluate differences between conservation varieties ($n = 60$) and modern cultivars ($n = 36$) (ns = p -value > 0.05 ; * = p -value < 0.05 ; ** = p -value < 0.01 , *** = p -value < 0.001 , **** = p -value < 0.0001). In b) each boxplot represents 12 biological replicates ($n = 12$), means not sharing any letter are significantly different (multiple comparison with Šidák correction, $\alpha = 0.05$). Conservation varieties are represented by shades of blue, while modern varieties are represented by shades of red. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

significantly higher than all other conservation varieties, but comparable to Bidì (Fig. 6b). Some variability was present between environments: Bidì, Perciasacchi and Russello had %UPP significantly higher than Simeto in Caltavuturo (2021); in Selinunte (2021) the %UPP was higher in Perciasacchi in comparison to Orizzonte; Bidì %UPP was always comparable to the three modern varieties apart from Selinunte (2021), where its content was significantly lower than Iride (Supplementary Fig. S27).

2.6. Heritability

To estimate the phenotypic variance attributable to the genotype, broad-sense heritability (h^2) was computed for each trait (individual ATIs, total ATI, fructans and %UPP) using the method reported in Piepho and Möhring (2007) (Table 3).

For three ATIs (0.28, WTI and WASI), the h^2 was higher than 0.99. The majority of ATIs had an h^2 higher than 0.90, except CM16 ($h^2 = 0.891$) and CM3 ($h^2 = 0.828$), while total ATI had a low h^2 , equal to 0.539. High values of heritability were also found for %UPP and fructans ($h^2 = 0.973$ and $h^2 = 0.925$, respectively).

3. Discussion

In recent years, there has been a renewed interest in wheat “conservation” varieties. Their preservation is important for biodiversity and as valuable sources of genes for breeders (Fiore et al., 2022). Additionally, consumers tend to prefer products made from conservation varieties, because they are perceived as more natural, traditional and healthier. The health aspect is rather controversial: while several studies demonstrated that conservation varieties are not safe for people suffering from CD, some studies supported the theory that their consumption could help to ameliorate NCWS and IBS symptoms (Brouns et al., 2022; Spisni et al., 2019). Knowledge on the composition of the kernel of these varieties, in comparison to the modern ones, is essential to validate these theories and to better understand the physiological mechanisms behind them.

At the moment, ATI and fructans are the wheat grain components suspected to worsen the symptoms of NCWS and IBS. Different studies based on mass spectrometry evaluated the differences in ATI content and composition between common wheat, durum wheat and other wheat relatives including spelt, emmer and einkorn (Asledottir et al., 2025; Call et al., 2020; Geisslitz et al., 2020; Sielaff et al., 2021). Two different research groups quantified ATI in common wheat cultivars released between 1890 and 2013, both reaching the same conclusion that breeding did not change ATI content and composition (EL Hassouni et al., 2021; Geisslitz et al., 2023). Recently, Jahn et al. (2025) evaluated ATI content and alpha-amylase inhibitory activity of 14 German common wheat landraces and 6 modern common wheat cultivars and found no significant differences. However, to date, there are no data available about the ATI content and composition of durum/tetraploid wheat

conservation varieties.

Also for fructans, the majority of studies focused on differences between common wheat and durum wheat and their relatives (Brandolini et al., 2011; Kulathunga and Simsek, 2022; Verspreet et al., 2012). Ribeiro et al. (2022) quantified fructans in a large set of genotypes, including common wheat, durum wheat modern varieties and landraces, and spelt, identifying significant differences between modern durum wheat varieties and landraces. No data are available at the moment for Sicilian conservation varieties.

In this study, we evaluated the ATI and fructan content of five Sicilian conservation varieties in comparison to three modern varieties in a multi-environment trial. The varieties were grown in two different locations in Sicily for three consecutive years, in order to study the environmental influence on the accumulation of these compounds. Additionally, the %UPP was calculated to provide a predictive assessment of technological quality. For each trait, two different linear mixed effect models were computed: one to study the effects of the variety and the environment, the other to study the effect of the “type” (conservation varieties or modern cultivar) and the environment.

A targeted LC-MS/MS method based on stable isotope dilution analysis (SIDA) was used to quantify the 13 major ATI. All samples analysed showed a content of CM1, CM17 and WCI below the LOD. This was expected and concordant with other studies, because all these ATIs are encoded by the D genome of common wheat and, thus, are not present in tetraploid wheat (Geisslitz et al., 2020, 2022).

ATI 0.28 was not present in all the varieties, as Bidì, Perciasacchi and Simeto had a content below the LOD. Geisslitz et al. (2020) found the presence of 0.28 only in two of the eight modern durum wheat cultivars analysed and in all the emmer cultivars. Simonetti et al. (2022) reported the amplification of the gene encoding ATI 0.28 only in one durum wheat cultivar and not in the other three tetraploid wheat cultivars analysed (one emmer, one *turanicum* and one durum). Camerlengo et al. (2020) used genome editing to silence ATI CM3 and CM16 in the durum wheat cultivar Svevo and found the presence of ATI 0.28 through ELISA and RT-PCR, in the edited lines but, surprisingly, not in the wild-type. On the same lines, targeted and non-targeted mass spectrometry approaches were used to specifically quantify ATI and, this time, ATI 0.28 was found in both wild-type and edited lines (Geisslitz et al., 2022). Overall, it seems that not all the tetraploid wheat genotypes express ATI 0.28, and, in some cases, the relative gene is missing. Further studies are needed to fully understand the expression and the role of 0.28 in tetraploid wheat. In our study, the lack of ATI 0.28 did not influence the total ATI content, despite constituting approximately 3–6 % of the total in the varieties that contain it.

ANOVA results indicated that for all ATIs and for total ATI there is a significant effect of the interaction between variety and environment. Similar results were obtained for the interaction between “type” and environment, except for 0.53 and 0.28. In these cases, only the main effects were significant. There is poor knowledge on how environmental conditions affect ATI accumulation in tetraploid wheat. Prandi et al. (2013) reported a significant effect of both genotype and environment on CM3 content in three durum wheat cultivars. Simonetti et al. (2022a, b) measured the alpha-amylase and trypsin inhibitory activities of different varieties belonging to einkorn, emmer, khorasan, spelt and common wheat and found a significant effect of the interaction of genotype and environment for alpha-amylase inhibition, but not for trypsin inhibition. Anyway, the absence of correlation between ATI content and alpha-amylase inhibitory activity was already observed in two previous studies (Jahn et al., 2023, 2025). Jahn et al. (2025) found significant effect of the harvest year on ATI accumulation in bread wheat, while variety and the interaction between variety and harvest year was not significant. Our results suggest that environmental factors have a significant impact on ATI accumulation in tetraploid wheat.

The WTAI CM3, CM2 and CM16 were the most abundant in the varieties analysed, followed by WDAI 0.19 and 0.53 and WMAI 0.28 (when present). Other protease inhibitors such as CMX1/2/3, WTI and WASI

Table 3
– Heritability (h^2) of each trait analysed (individual ATIs, total ATI, fructans and %UPP).

Trait	h^2
CM3	0.828
CM2	0.933
CM16	0.891
0.53	0.963
0.19	0.913
0.28	0.999
CMX1/2/3	0.989
WASI	0.993
WTI	0.998
Total ATI	0.539
Fructans	0.925
%UPP	0.973

constitute a minor fraction of the total ATI. The ATI composition found in this work was similar to the one obtained by Geisslitz et al. (2020) for durum wheat. Although significant differences were found for each ATI between conservation varieties and modern cultivars, except for CM3, no evident differences in general ATI composition were found. Conservation varieties had a higher content of CM2, CM16, 0.19, CMX 1/2/3, WASI and WTI, while modern varieties had a higher content of 0.53 and 0.28. Despite this, the total ATI content was not significantly different between conservation varieties and modern cultivars.

The most noticeable differences between varieties were found for the less represented ATI. In particular, Timilia reste bianche and Timilia reste nere showed a WTI content significantly lower than other varieties, $7.74 \pm 0.71 \mu\text{g/g}$ and $9.90 \pm 0.90 \mu\text{g/g}$, respectively. In comparison, the other varieties had a mean content between $75.63 \pm 2.46 \mu\text{g/g}$ (Iride) and $128.73 \pm 5.57 \mu\text{g/g}$ (Perciasacchi). Geisslitz et al. (2020) found huge variability in the content of WTI, ranging from $13.74 \mu\text{g/g}$ to $136.87 \mu\text{g/g}$ in durum wheat. On the contrary, Timilia reste bianche and Timilia reste nere had the significantly highest content of WASI.

Perciasacchi, the only *T. turanicum* (khorasan) variety included in this study, had the highest content of CMX1/2/3 and WTI, both belonging to the category of trypsin inhibitors (Simonetti et al., 2022). tested the trypsin inhibitory activity in one *T. turanicum* genotype, QK-77, and found no evident difference in comparison to other tetraploid wheats. Further studies could focus on the trypsin inhibitory activity of Perciasacchi.

The Pearson method was used to study correlations among each individual ATI. A strong positive correlation between CM2 and CM3 was found ($r = 0.73$, $p\text{-value} < 0.05$). Gomez et al. (1989) supposed that tetrameric ATIs in *T. turgidum* are composed of two subunits of CM3, one of CM2 and one of CM16. Anyway, we did not find strong correlations between CM3 and CM16 ($r = 0.26$, $p\text{-value} < 0.05$) and between CM2 and CM16 ($r = 0.46$, $p\text{-value} < 0.05$). In previous studies, stronger correlations among ATIs have been reported compared to our findings. Jahn et al. (2023) reported a strong correlation between CM2, CM3, CM16 and CM17 ($r > 0.85$) in emmer, durum wheat, spelt and common wheat. EL Hassouni et al. (2021) found a strong correlation between all the CM-types (CM1, CM2, CM3, CM16, CM17) in common wheat, with r between 0.69 and 0.87. Geisslitz et al. (2023) quantified ATIs in 60 different varieties of common wheat and they also found strong correlations between all the CM-types ATIs ($r > 0.77$). Also Jahn et al. (2025) found a strong correlation for these ATIs ($r > 0.83$) in common wheat landraces and modern cultivars.

High broad-sense heritability ($h^2 > 0.82$) was found for all ATIs except for the total ATI content ($h^2 = 0.539$). These results are similar with the ones reported in Geisslitz et al. (2020) for durum wheat, except for 0.28 and 0.53, for which they found low heritability ($h^2 = 0.14$ and $h^2 = 0.00$, respectively), and total ATI ($h^2 = 0.90$). In their study, eight German modern durum wheat cultivars were cultivated in three locations during one growing season. Lack of data on other growing seasons could have led to an underestimation of the heritability. Moreover, the intrinsic genetic variability of the population under study can influence the heritability computation.

Overall, our data indicates that Sicilian conservation varieties, when grown in their local environment, are not characterized by a lower content of ATI in comparison to modern cultivars. There were differences in the content of minor ATIs, however, the limited knowledge regarding their bioactivity precludes speculation on a potentially reduced capacity to induce adverse reactions. In our study, environmental conditions had a high impact on ATI accumulation. However, while high heritability was found for all individual ATI, it was not the case for total ATI, suggesting a good opportunity for using these varieties in breeding programs aimed at modifying ATI composition.

Conservation varieties showed a significantly lower fructan content in comparison to modern varieties. This was mostly influenced by the lower content in Bidi and Russello in comparison to all the other varieties. In fact, all the varieties showed values between 1.50 % (w/w) and

1.63 % (w/w) while Bidi and Simeto had 1.29 % (w/w) and 1.24 % (w/w), respectively. Further studies are needed to understand if this reduction is related to health properties of wheat products.

The effect of the interaction between variety and environment was significant for both the models. Fructans are accumulated in wheat in response to various abiotic stresses, so it was not surprising to find significant environmental effects (Bie et al., 2012; Cimini et al., 2015; Kawakami et al., 2008; Yáñez et al., 2017; Zhang et al., 2015). However, we also found high heritability for this trait ($h^2 = 0.925$), in agreement with previous studies (Huynh et al., 2008; Ziegler et al., 2016).

One main objective of wheat breeding programs is the improvement of technological quality, mostly through the selection of the best combination of glutenin subunits (De Santis et al., 2017; De Vita et al., 2007). For this reason, modern varieties tend to have better technological properties than conservation varieties, representing a limitation to the utilization of the latter (Fois et al., 2011; Spina et al., 2023). In this work, we used the analysis of %UPP, a rapid and convenient method based on size exclusion chromatography, to predict technological quality (Edwards et al., 2007; Gupta et al., 1993; Sissons et al., 2005). As expected, modern varieties showed a %UPP significantly higher than conservation varieties. Interestingly, Bidi showed a %UPP comparable to modern varieties in almost all environments. Bidi, unlike the other conservation varieties examined in this study, is a historical cultivar, selected at the beginning of 19th century from a Tunisian landrace. In a precedent work, we measured %UPP in eight Sicilian conservation varieties (including those used in this study) in comparison to one modern durum wheat variety, Saragolla, obtaining slightly different results (Palombieri et al., 2024). In that work the %UPP of Bidi was comparable to Saragolla only in one year and the %UPP of Russello was comparable to Saragolla in both years. Such differences can be explained with the different environmental conditions in the two studies. In fact, we found a significant effect of the interaction between variety and environment and between “type” and environment. As expected, the heritability of %UPP was high ($h^2 = 0.973$).

In conclusion, this is the first study that reports differences in ATI composition and fructan content between Sicilian conservation varieties and modern cultivars. Further studies are necessary to ascertain whether the differences observed have biological relevance and could contribute to the effective health improvement of wheat products.

4. Methods

4.1. Grain samples

Five tetraploid wheat conservation varieties (Bidi, Perciasacchi, Russello, Timilia reste bianche, Timilia reste nere) and three modern cultivars (Iride, Orizzonte, Simeto) were cultivated for three years (2020–2021, 2021–2022, 2022–2023) across two different locations in Sicily: Caltavuturo (Palermo), a locality included in the Madonie Park and in the hinterland of the Himera valley at 635 m above sea level ($37^{\circ}47'43''\text{N}$ $13^{\circ}54'20''\text{E}$), and Selinunte (Trapani), a locality in the South-Western coast of Sicily, a few meters above sea level ($37^{\circ}54'20''\text{N}$ $12^{\circ}54'07''\text{E}$).

Kernels of the different conservation varieties were collected from Cinozoo 3R srl (Bisacquino, Palermo, Italy), a certified maintainer of these varieties. Before the harvest, the correspondence of the spikes morphology with the UPOV descriptors was evaluated. Off-type spikes were discarded. Kernels of Iride (Società Produttori Sementi, Argelato, Italy), Orizzonte (Fratelli Menzo, Piazza Armerina, Italy) and Simeto (Società Italiana Sementi, San Lazzaro di Savena, Italy) were collected from certified seed resellers. These three varieties were chosen as they ranked among the top ten most cultivated varieties in Sicily during the period 2018–2020 (data provided by Consorzio di Ricerca Ballatore).

All the varieties belong to *T. turgidum* ssp. *durum*, except for Perciasacchi, which belongs to *T. turgidum* ssp. *turanicum*. A randomized complete block design with three replications was implemented at each

site. Wheat samples were harvested, and grains were milled in a Cyclotec 1093 (FOSS, Denmark) equipped with a 0.5 mm sieve.

4.2. ATI extraction and analysis

Thirteen ATIs were extracted according to Geisslitz et al. (2020) and quantified using the same targeted LC-MS/MS method based on SIDA reported in Jahn et al. (2025). The calculation of the content of individual ATI was based on Geisslitz et al. (2020). In Table 4 the peptides and internal standards used for each ATI quantification with their LOD and limit of quantification (LOQ) are reported. The total ATI content was calculated as the sum of all the ATIs.

Peptide separation was achieved with a Vanquish UHPLC system (Thermo Fisher Scientific, Waltham, MA, USA) equipped with an Aeris 1.7 μm PEPTIDE XB-C18 (100 Å, 150 × 2,1 mm, Phenomenex, Aschaffenburg, Germany) using formic acid (0.1 %, v/v) in water as solvent A and formic acid (0.1 %, v/v) in acetonitrile as solvent B. Gradient, injection volume and column temperature were the same of Jahn et al. (2025). Calibration was performed exactly as reported by Geisslitz et al. (2020). Scheduled parallel reaction monitoring was performed with a Q Exactive Plus Orbitrap (Thermo Fisher Scientific) and it was operated as described in Jahn et al. (2025). Peak area integration was performed using Skyline (v 23.1, MacCoss Lab Software, University of Washington, Seattle, WA, U.S.A.) with manual verification of automated peak integration. Two technical replicates were analysed for each sample.

4.3. Fructan quantification

Total fructan content was determined using the Fructan Assay Kit K-FRUC (Megazyme, Ireland). Two technical replicates were analysed for each sample.

4.4. Unextractable polymeric protein (%UPP)

The percentage of unextractable polymeric protein was calculated using the method reported by Palombieri et al. (2024). A probe-type sonicator SONICS Vibracell model VC 50T (power 50 W, frequency 20 KHz) was used for 15 s for the suspension of unextractable polymeric proteins. The analysis was conducted in a Smartline system (Knauer, Germany) equipped with a TSKgel SuperSW3000 column (300 mm × 4.6 mm, Tosoh Bioscience, Japan). Two technical replicates were analysed for each sample.

4.5. Statistical analysis

For each variable measured, a linear mixed effects model was applied using “variety”, “environment” (the combination of location and year) and their interactions as fixed effects and blocks within the environment as random effects (“Variety x Environment” model). A two-way ANOVA (α = 0.05) was used to assess the effect of each factor on each measured variable. Multiple comparisons were performed on estimated marginal means with Šidák correction.

A second linear mixed effects model was fitted for each variable measured using the factor “breeding type” (modern cultivar or conservation variety) instead of “variety” and a two-way ANOVA was performed (“Type x Environment” model). Bidi, Perciasacchi and Simeto were excluded from the “Type x Environment” model of ATI 0.28, because the content was below the LOD. Timilia reste bianche and Timilia reste nere were excluded from the “Type x Environment” model of WTI because the content was really low, close to the LOD.

Pairwise *t*-test on estimated marginal means (α = 0.05) was performed to analyse differences in ATI composition between modern cultivars and conservation varieties.

Heritability was calculated as $H^2 = \frac{\sigma_g^2}{\sigma_g^2 + \frac{\sigma_{BLUE}^2}{2}}$ where σ_g^2 is the genetic

Table 4
– Peptides and internal standards used for the quantification of each ATI. C, S-carboxamidomethylcysteine; *P, proline ([¹³C]₅, [¹⁵N]); *V, valine ([¹³C]₅, [¹⁵N]); *K, lysine ([¹³C]₆, [¹⁵N]₂); *R, arginine ([¹³C]₆, [¹⁵N]₄) *G, glycine ([¹³C]₂, [¹⁵N]); *L, leucine ([¹³C]₆, [¹⁵N]).

Peptide/ Internal standard	ATI	Amino acid sequence	LOD (μg/g)	LOQ (μg/g)
P2/IS2	0.28	LTAASVPEVC*K	6.35	21.16
P3/IS3	0.19 + 0.53	LQCNGSQVPEAVL*R	1.35	4.51
P5/IS5	0.53	EHGVSEGGAGTGAFPSG*R	11.27	37.57
P6/IS6	CM1	SDPNSSVL*K	0.45	1.52
P8/IS8	CM2	TSDPNSGVL*K	2.55	8.51
P9/IS9	CM3	YFIALPVPSQPVDP*R	1.84	6.14
P11/IS11	CM16	DYVEQQAC*R	6.98	23.27
P13/IS13	CM17	NYVEEQAC*R	1.97	6.58
P14/IS14	WASI	HVITGPV*R	1.89	6.31
P16/IS16	CMX1/ 2/3	EFIAGIVG*K	0.57	1.9
P18/IS18	WCI	AFPPSQSQGGPPQPPLAP*R	6.28	20.92
P20/IS20	WTI	LEGVPEGCT*R	1.8	5.99

variance and $\bar{v}_{\Delta}^{BLUE}$ is the mean variance of a difference of two genotypic BLUEs (Piepho and Möhring, 2007).

Pearson correlation between individual ATIs was computed and visualized using the R package *heatmap* (Kolde, 2019).

All the statistical analyses were performed with R v4.4.0 using the packages *lme4* and *emmeans* (Bates et al., 2015; Lenth, 2024; R Core Team, 2024).

CRediT authorship contribution statement

Marco Bonarrigo: Writing – review & editing, Resources, Project administration. **Sabrina Geisslitz:** Writing – review & editing, Methodology, Data curation. **Katharina Anne Scherf:** Writing – review & editing, Methodology. **Bernardo Messina:** Writing – review & editing, Writing – original draft, Software, Investigation, Formal analysis, Data curation. **Giuseppe Russo:** Writing – review & editing, Resources, Project administration. **Francesco Sestili:** Writing – review & editing. **Stefania Masci:** Writing – review & editing, Supervision, Project administration, Investigation, Funding acquisition, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jcs.2025.104190>.

Data availability

Data will be made available on request. Mass spectrometry data of the LC-MS/MS experiments are publicly available on Panorama Public (<https://panoramaweb.org/tFDGort/>).

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As part of the CIRCE project, we also genetically characterized the five conservation varieties. To this end, we collected approximately 140 accessions from various farmers responsible for their maintenance. Additionally, we included several accessions of the conservation variety Ruscia, given its similarity to Russello.

We also included in the dataset several of the most widely cultivated modern varieties in Sicily, as well as some landraces and historical varieties to serve as a reference for comparison.

The aim of this work was to study the diversity between the different conservation varieties and to develop a DNA-based traceability method, to apply throughout the supply chain.

6 Chapter IV - Assessing genetic diversity and developing traceability tools for Sicilian tetraploid wheat conservation varieties supply chain

6.1 Introduction

Durum wheat (*Triticum turgidum* L. ssp. *durum* (Desf.) Husn., $2n=4x=28$ AABB genome) is an important crop worldwide, with an annual production of about 37 million tonnes (Božek et al., 2021). It is particularly cultivated in the Mediterranean area, where it is typically used for production of pasta, couscous, bulgur and some types of bread, but its cultivation extends also to Northern Europe Countries such as Denmark, Finland and Sweden. This crop is especially important in Italy, which is in fact one of the largest producers in the world and the first country for pasta production (Blanco, 2024).

The historical importance of this crop for Italy is evidenced by the fact that the first breeding programs started there, at the beginning of the 1900s. In that period, durum wheat cultivation was based mainly on landraces adapted and evolved in the southern regions of Italy, especially in Sicily. First breeding programs involved the selection of pure lines from these landraces and from landraces originating from North Africa. The most famous variety developed in this way was Cappelli (*syn.* Senatore Cappelli), selected by Nazareno Strampelli from the Tunisian landrace Jean Retifah and released in 1915; although (E. De Cillis, 1927) and some recent molecular studies suggest that it was selected from Bidì or Mahmoudi, two other Tunisian landraces (Fiore et al., 2019; Marzario et al., 2018; Taranto et al., 2020). The selection process brought the release of different varieties, most belonging to the *mediterraneum* type, until the mid of the 19th century, when breeders started to cross them with the *syriacum* type landraces (Giunta et al., 2007). The crosses were characterized by semi-dwarfism and increased earliness, traits that contributed to the replacement of old varieties and landraces. Finally, Green Revolution led to the introgression of *reduced height* (*Rht*) dwarfing genes, enhancing lodging resistance and causing a huge yield improvement (De Vita et al., 2007).

For many years, landraces and historical cultivars have been maintained only by farmers and research institutes, with the risk of contamination or losing them. In recent years, both consumers and the scientific community have shown renewed interest in these ancient genotypes. Modern breeding efforts have substantially increased the productivity and agronomic performance of durum wheat. However, this progress has often come at the expense of genetic diversity, as intensive selection and the repeated

use of a narrow genetic base have led to the erosion of valuable alleles present in traditional varieties and landraces. (Mazzucotelli et al., 2020; Taranto et al., 2020). Moreover, these varieties represent a useful resource of valuable alleles that need to be exploited yet, especially those associated with resistance to biotic and abiotic stresses (Campanella & Petralia, 2022; Corinzia et al., 2024; Palermo et al., 2024; Ruisi et al., 2015; Scandurra et al., 2024; Scavo et al., 2022).

To promote the preservation of their biodiversity, the Italian government, implementing the EU directive 2008/62/CE, with the Legislative Decree No. 149 of 2009 (now repealed and replaced by Legislative Decree No. 20 of 2021) permitted the registration of “conservation varieties”, defined as “landraces and varieties which are naturally adapted to local and regional conditions and threatened by genetic erosion”, in the National catalogue of agricultural species. Since 2009, twenty-eight durum wheat conservation varieties have been inscribed in the Catalogue and twenty-four of them are Sicilian.

Sicily has played a pivotal role in the history of durum wheat cultivation and remains the second-largest Italian region for its production. Here, historical varieties are cultivated in marginal areas under low-input farming systems, partly due to their yield stability. The products derived from these varieties are positioned in high value and niche markets, where attributes such as unique sensory qualities, nutritional benefits, cultural heritage, and sustainable production practices are highly valued by consumers (De Vita et al., 2010; Varia et al., 2021).

One of the major challenges in the conservation varieties supply chain is the risk of genetic contamination, which can arise from different factors, such as unintentional seed mixing before the sowing or during the harvest, errors in morphological identification or intentional fraud.

One of the main methods for identifying wheat varieties involves analysing the electrophoretic patterns of glutenin subunits and gliadins through SDS-PAGE (sodium dodecyl sulphate-poly-acrylamide gel electrophoresis) and A-PAGE (acid-poly-acrylamide gel electrophoresis) (Bushuk & Zillman, 1978; Lawrence & Shepherd, 1980; Shewry et al., 1978). Visioli et al. (2021) used SDS-PAGE of glutenin subunits to analyse seed batches and commercial flours of Sicilian conservation varieties labelled as mono-

varietal and found in all the cases they detected varying degrees of genetic contamination. In a previous work, we demonstrated that gliadin and glutenin electrophoretic patterns can be used to discriminate 21 Sicilian conservation varieties, with the only exception of Bidi, Cappelli and Capeiti 8, that resulted undistinguishable (Palombieri et al., 2024).

Although these techniques are still widely used in wheat research due to their simplicity and cost-effectiveness, the analysis of DNA polymorphisms remains the most effective and high-performance strategy for studying genetic diversity. The earliest wheat genotyping methods were based on simple sequence repeats (SSRs) (Cooke et al., 2003; Pasqualone et al., 2010; Perry, 2004). Advancement in next generation sequencing techniques permitted the discovery of numerous single nucleotide polymorphisms (SNPs) and the development of different DNA microarray platforms (Allen et al., 2017; Burridge et al., 2024; Cavanagh et al., 2013; Rimbart et al., 2018; S. Wang et al., 2014; M. O. Winfield et al., 2016). All these platforms were originally designed on common wheat (*Triticum aestivum* L.) but they have been also used extensively to study durum wheat genetic diversity (Ganugi et al., 2021; Maccaferri et al., 2019; Mazzucotelli et al., 2020; Roncallo et al., 2021; Taranto et al., 2020; Velimirović et al., 2023).

Some authors used these SNP microarrays to study Sicilian tetraploid wheat conservation varieties. Fiore et al. (2019) used the Illumina Wheat 90K microarray to analyse 27 Sicilian tetraploid landraces from the collection of the “Experimental Sicilian Station for Durum Wheat” (ESS) of Caltagirone (Catania), together with two historical varieties (Cappelli and Trinakria) and two modern varieties. In a recent work Fiore et al. (2022) used the same microarray on a collection that included 104 accessions of *T. turgidum* subspecies, mostly consisting of Sicilian landraces. Also, in this case they were retrieved from ESS collection. Taranto et al. (2022) collected 357 accessions of Timilia reste nere and Russello from Sicilian farmers and preserved them following three generations of Single Seed Descendent (SSD) at the Research Centre for Cereal and Industrial Crops (CREA-CI) of Foggia (Italy). Then, the accessions were genotyped using the Illumina iSelect 15K wheat SNP array (TraitGenetics GmbH, Gatersleben, Germany) to analyse them.

Traceability DNA-based techniques are widely used in food of plant origin supply chains (Lanubile et al., 2024). Over the years, different approaches have been developed for durum wheat varietal identification. SSR markers have been successfully used for samples of seeds, bread and pasta (Fanelli et al., 2023; Pasqualone et al., 2010; Perry, 2004). SNPs have been exploited using a wide range of techniques, as Denaturing High Performance Liquid Chromatography (DHPLC), TaqMan genotyping, loop-mediated isothermal amplification reaction (LAMP) and Digital PCR (Cibecchini et al., 2020; Giancaspro et al., 2016; Lee et al., 2023; Morcia et al., 2021).

The aim of this work was to genetically characterize different accessions of six Sicilian tetraploid wheat conservation varieties (Bidì, Ruscia, Russello, Perciasacchi, Timilia reste bianche and Timilia reste nere) and to develop a DNA-based system to perform traceability through supply chain. These accessions were obtained from custodian farmers, who are actively involved in their conservation and maintenance.

For genetic characterization we performed SNP genotyping using the Wheat 7K Illumina Infinium microarray (Traitgenetics GmbH, Gatersleben, Germany), a platform containing 6707 high polymorphic SNPs selected from previous microarrays.

The resulting data were used to assess the genetic differentiation among the varieties and to identify a subset of SNPs able to discriminate efficiently them.

6.2 Methods

6.2.1 Plant materials

A total of 181 accessions of tetraploid wheat were analysed in this work, of which 141 accessions of Sicilian tetraploid wheat were collected directly from farmers. In each farm, varieties were classified by the morphology of the spike and two or three spikes for each type were collected. In total, we analysed 20 accessions of Bidì, 31 accessions of Perciasacchi, 15 accessions of Ruscia, 24 accessions of Russello, 28 accessions of Timilia reste bianche and 23 accessions of Timilia reste nere. Additionally, we included 16 durum wheat modern varieties, 9 durum wheat old cultivars and 15 tetraploid wheat landraces from various sources. The complete accession list is reported in

Supplementary Table 1, while Table 1 presents a brief description of the analysed varieties and the corresponding number of accessions.

Table 1 – List of the analysed varieties and their corresponding number of accessions. LR = landrace, OC = old cultivar, MC = modern cultivar.

Variety	Breeding type	Subspecies	N° Accessions	Description	Pedigree
Bidi	OC	<i>durum</i>	20	Sicilian old cultivar	Selection from the Tunisian landrace Mahmoudi
Anco Marzio	MC	<i>durum</i>	-	Modern cultivar	Stot//Altar 84/ ALD
Antalis	MC	<i>durum</i>	-	Modern cultivar	-
Aureo	MC	<i>durum</i>	-	Modern cultivar	Kofa/Svevo
Bronte	MC	<i>durum</i>	-	Modern cultivar	Berillo/Latino
Capeiti 8	OC	<i>durum</i>	2	Old Italian cultivar released in 1955, registered as conservation variety.	Eiti 6/Cappelli
Cappelli	OC	<i>durum</i>	5	Old Italian cultivar released in 1915	Selection from the Tunisian landrace Jenah Rhetifah
Duilio	MC	<i>durum</i>	-	Modern cultivar	Cappelli//Anhinga/Flamingo
Emilio Lepido	MC	<i>durum</i>	-	Modern cultivar	Orobel// Arcobaleno/Svevo
Etrusco	LR	<i>turanicum</i>	5	Turanicum landrace	-
Furio Camillo	MC	<i>durum</i>	-	Modern cultivar	Iride/Claudio
Graziella Ra	LR	<i>turanicum</i>	1	Turanicum landrace	-
Iride	MC	<i>durum</i>	-	Modern cultivar	Altar 84/Ionio
Isola	MC	<i>durum</i>	-	Modern cultivar	Rusticano/Platani
K26	MC	<i>durum</i>	-	Modern cultivar	Spontaneuos mutant derived from Simeto/Colosseo
QK-77	MC	<i>turanicum</i>	-	Turanicum cultivar released in 1990	
Mahmoudi	OC	<i>durum</i>	2	Tunisian landrace	-
Marco Aurelio	MC	<i>durum</i>	-	Modern cultivar	Orobel//Arcobaleno/Svevo
Opera	MC	<i>durum</i>	-	Modern cultivar	Duilio/unspecified CIMMYT line
Orizzonte	MC	<i>durum</i>	-	Modern cultivar	Rusticano/Simeto

Perciasacchi	LR	<i>turanicum</i>	32	Sicilian Turanicum landrace	-
Redidenari	MC	<i>durum</i>	-	Modern cultivar	Cofa/N185
Ruscia	LR	<i>durum</i>	15	Sicilian landrace	-
Russello	LR	<i>durum</i>	26	Sicilian landrace	-
Samarcanda	MC	<i>durum</i>	-	Modern cultivar	Capeiti 8/Rusticano
Saragolla lucana	LR	<i>turanicum</i>	3	Turanicum landrace	-
Simeto	MC	<i>durum</i>	-	Modern cultivar	Capeiti 8/Valnova
Timilia reste bianche	LR	<i>durum</i>	28	Sicilian landrace	-
Timilia reste nere	LR	<i>durum</i>	23	Sicilian landrace	-
Trinakria	OC	<i>durum</i>	2	Old Italian cultivar released in 1970	B14/Capeiti 8

The collection was composed by 139 accessions of *T. turgidum* spp. *durum* and 42 of *T. turgidum* ssp. *turanicum*. Accessions were divided into landraces (LR), old (OC) and modern cultivars (MC) based on their year of release and their constitution.

6.2.2 DNA extraction and SNP genotyping

One seed from each individual accession was germinated and leaves were sampled for DNA extraction. DNA extraction and SNP genotyping were performed by Traitgenetics GmbH (Gatersleben, Germany). For SNP genotyping, the Illumina Infinium 7K wheat microarray (Traitgenetics GmbH, Gatersleben, Germany) was used, containing 6713 SNP markers.

6.2.3 Data quality control

Genotyping data were firstly converted into an HapMap file, adding the physical position of each probe on durum wheat genome (Svevo.v1), using R v4.4.3 (R Core Team, 2024). All the probes that did not map were removed. Then, the HapMap file was converted into a Plink file using TASSELL v.5 (Bradbury et al., 2007). Plink v1.9 and Plink v2.0 were used for initial data quality filtering (Chang et al., 2015). Probes mapping on multiple positions were removed using the plink2 function '--rm-dup exclude-all'. Probes mapping on the same physical position were identified (plink --list-duplicate-vars ids-only) and

removed. Then, we excluded markers with missing call rates higher than 5% (plink --geno 0.05) and samples with missing call rates higher than 10% (plink --mind 0.1).

6.2.4 Sample filtering

To identify duplicates accessions and possible misidentifications, the function *mlg.filter* of the R package *poppR* v2.9.6 was used (Kamvar et al., 2015). We used this function to collapse unique multilocus genotypes using a nearest neighbour clustering method calculated on Hamming distance. For this analysis missing data were removed. Accessions of a variety that clustered together with accessions of another variety were removed, because considered misidentifications. For each cluster, only one accession was kept, choosing the one with less missing data. If a cluster contained accessions from multiple varieties, at least one accession from each variety was retained.

To identify more misidentifications, we calculated identity-by-state (IBS) using the plink function '--distance square ibs' and we produced a clustered heatmap representing IBS results using the R package *pheatmap* v1.0.12 (Kolde, 2019). Accessions of a variety that were in a separate cluster than the other accessions of the same variety were removed.

6.2.5 Population structure and admixture analysis

Markers with a minor allele frequency (MAF) lower than 1% were removed using the plink function '--maf 0.01'. Then, linkage disequilibrium (LD) pruning was made using a r^2 threshold of the 80%, a window size of 50 kb and a step size of 5 (plink --indep-pairwise 50 5 0.8).

The structure of the population and the individual admixture proportions were calculated using the software PopCluster v1.4 (J. Wang, 2024). Best number of population clusters (K value) was determined performing 100 replicate runs (K range from 2 to 20) using two different built-in K estimators: the second order rate of change of the estimated log-likelihood and the F statistics. Then, 1000 replicate runs were made with the ideal number of clusters. Default settings were used except for likelihood scaling, that was set as medium.

The admixture plot was produced in R using the package *ggplot2* v3.5.1 (Wickham, 2016).

Pairwise fixation index (F_{ST}) between populations was calculated using PopCluster and plotted using the R package *corrplot* v0.95 (Wei & Simko, 2024).

6.2.6 Diversity analysis

Multidimensional scaling was computed on the pruned SNP dataset using the plink function '--cluster --mds-plot'. The plot was produced using the R package *ggplot2* v3.5.1 (Wickham, 2016).

To study the phylogenetic relationships between the accessions, a neighbor-joining tree on Hamming distance was computed. Hamming distance was calculated using the plink function '--distance square' while the neighbor-joining tree was estimated using the R package *ape* v5.8 (Paradis & Schliep, 2019). Tree bootstrap values were calculated using *poppR* v2.9.6 (Kamvar et al., 2015). The tree was plotted using the R package *ggtree* v3.14.0 (Yu et al., 2017).

6.2.7 SNP statistics

The distribution of markers on the different chromosomes was plotted using the web server SRplot (D. Tang et al., 2023).

Nei's gene diversity (expected heterozygosity) and the number of private alleles were calculated using the R package *poppR* v2.9.6 (Kamvar et al., 2015). Analysis of molecular variance (AMOVA) between populations was computed using the same package. Significance testing was obtained through a randomization method with 1000 permutations implemented in the *randtest()* function of the R package *ade4* v1.7.23 (Thioulouse et al., 2018).

6.2.8 Traceability

A custom R script was developed to identify discriminant markers that are specific to a given variety or population. Briefly, starting from the dataset that had not been filtered for MAF and LD pruned, SNPs with a MAF of 0 within the accessions of the target variety/population were initially selected. This subset of SNPs was then assessed across the entire dataset to identify unique alleles (alleles exclusively present in the variety/population of interest). In cases where no unique alleles were detected, an alternative approach was implemented to identify a set of discriminant probes. First,

SNPs that were heterozygous or missing were removed. The MAF was calculated, and only those markers for which the target variety/population carried the minor allele were retained. Finally, SNPs with the lowest MAF values were sequentially selected until a sufficient number was obtained to distinguish the variety/population from all others.

KASP (Kompetitive Allele Specific Polymorphism) markers were designed using Polymarker v1.2.1 (Supplementary Table 2) (Ramirez-Gonzalez et al., 2015). KASP validation was performed on a BioRad CFX duet Real-Time PCR System (Bio-Rad, California, USA). DNA of an accession of each target variety/population was extracted using the method reported in Frittelli et al. (2025). Each KASP reaction was set as follows: 5 μ L of KASP-TF V5.0 2X Master Mix (LGC genomics), 0.14 μ L of KASP primer mix, 4.86 μ L of DNA. KASP primer mix was obtained mixing 30 μ L of 100 μ M common primer with 12 μ L of each 100 μ M allele-specific primer and 46 μ L of DNAase-free water. Cycling conditions suggested by TAQ manufacturer were used.

6.3 Results

6.3.1 SNP genotyping

6713 SNPs were characterized in 182 accessions of tetraploid wheat varieties using the Illumina Infinium 7K wheat microarray (Traitgenetics GmbH, Gatersleben, Germany). First step of the analysis was the quality filtering of the obtained markers. A total of 1126 SNPs that did not map on Svevo v.1 genome, map on multiple positions or map on the same position were removed. 444 SNPs were removed because their call rate was below the 95%, leaving a final dataset of 5143 variants. All the samples analysed had a call rate higher of 90%.

To remove duplicates or accessions that were not correctly identified, a multilocus genotype clustering approach was used. Clusters and the list of accessions that were retained or excluded from the analysis are reported in Supplementary Table 3. Clusters 1, 3, 4, 5, 6, 9, 10, 11, 12 and 13 contained multiple accessions of the same variety. In these cases, the choice of accessions to retain was based on the percentage of missing values. In cluster 2 one accession of Timilia reste bianche and one accession of Timilia reste nere were grouped together; given the likely misidentification, we retained only the accession with fewer missing data ("Timiliarb41B"). Several accessions belonging to

different *turanicum* varieties (Perciasacchi, Etrusco, Graziella Ra, Saragolla Lucana) clustered together in cluster 8; in this case we retained only one accession for each variety. Cluster 7 included four accessions of Bidì, all the accessions of Cappelli, two accessions of Timilia reste bianche and seven accessions of Timilia reste nere. The accessions belonging to “Timilia” varieties were considered misidentifications and removed, while one accession each of Bidì and Cappelli was retained.

A clustered heatmap based on Identity-by-State (IBS) distances among the remaining 126 accessions was generated to identify other potential misidentifications (Supplementary Figure 1). The accessions “Perciasacchi_13B”, “Ruscia_32B”, “Ruscia_32C” and “Timilia_rn_58B” clustered together with modern and old cultivars and were therefore excluded. Additionally, “Perciasacchi_10B”, “Russello_7” and “Bidì_1C” clustered separately from all the other accessions and were removed as well. The resulting dataset, consisting of 119 samples, was used for subsequently analyses.

SNP markers were filtered using a minor allele frequency (MAF) threshold of 5%, followed by linkage disequilibrium (LD) pruning with an r^2 threshold of 0.80. The final dataset was composed by 1645 high-quality SNPs distributed across the 14 chromosomes (Figure 12).

The number of SNPs within 1Mb window size

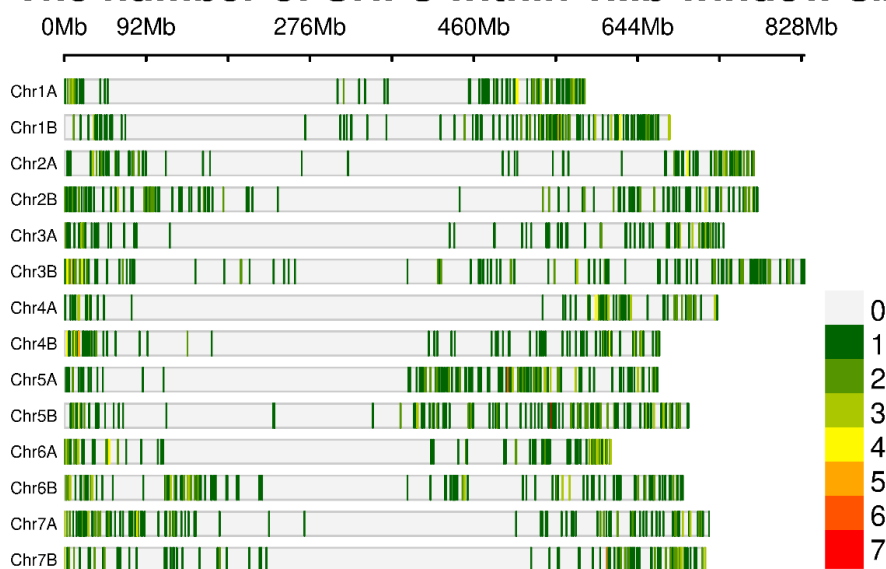


Figure 12 – Distribution of the 1645 high quality SNPs across the 14 chromosomes within 1 Mb window size.

The number of SNPs for each chromosome is reported in Supplementary Table 4. On average, the dataset contained 117 SNPs per chromosome. Chromosome 4A had the lowest number of SNPs (81), while chromosomes 2B and 5B had the highest (145 SNPs).

6.3.2 Population structure

Population structure was inferred and individual admixture proportions were estimated using an unsupervised likelihood method included in the software PopCluster. The optimal number of population clusters ($K=6$) was determined using a maximum likelihood clustering method under a mixture model. Then admixture proportions were calculated using an expectation maximisation algorithm. Every accession was considered belonging to a population if the relative admixture proportion was above 0.5. The admixture plot is reported in Figure 13 and the admixture proportion of each accession are reported in Supplementary Table 5.

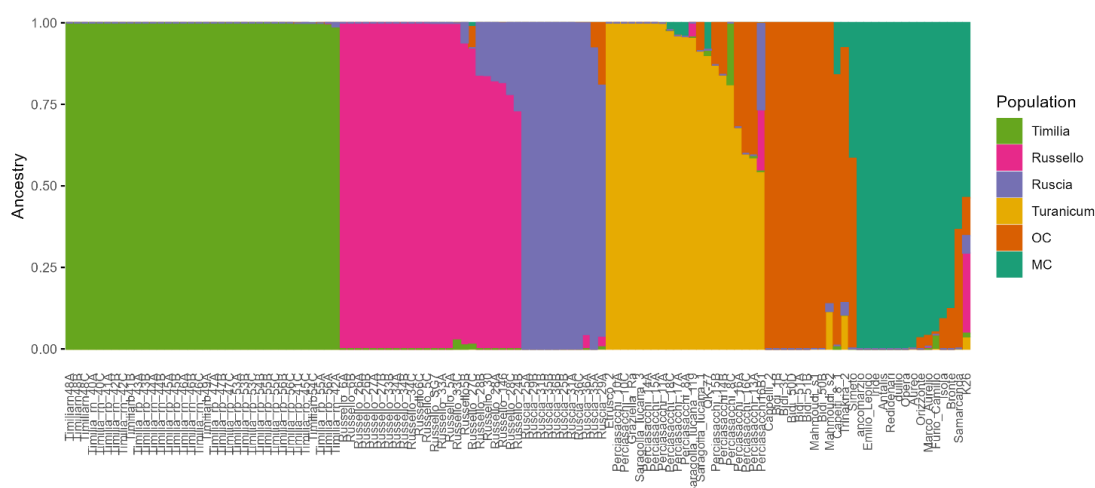


Figure 13 – Admixture plot ($K=6$) of 119 accessions analysed using 1645 markers. Each vertical bar represents one accession. In each bar, the color represents the ancestry (admixture proportion) relative to one of the six populations. OC = old cultivars, MC = modern cultivars.

The first cluster included all accessions of Timilia reste bianche and Timilia reste nere ($n=36$) and was therefore designed as the “Timilia” cluster (Figure 13, in green). These accessions exhibited minimal admixture with the other populations, with all accessions having an ancestry in this cluster > 0.987 .

The second cluster included all the Russello accessions ($n=24$), while third cluster included all the Ruscia accessions ($n=11$) (Figure 13, in pink and in purple respectively).

Some Russello accessions shared ancestry with the Ruscia cluster up to 0.269. All Ruscia accessions shared low ancestry with other populations.

All accessions belonging to *T. turgidum* ssp. *turanicum* (n=21) clustered together (Figure 13, in yellow). In this case, the population structure did not turn out to be so distinct, with 4 Perciasacchi accessions showing an ancestry lower than 0.7.

The fifth cluster (n=12) grouped all the accessions classified as “old cultivars” (OC, defined as the cultivars released before the 1970) along with the modern cultivar, Simeto (released in 1988) (Figure 13, in orange). Notably, Simeto is the oldest “modern cultivar” included in this study and one of its parental lines is Capeiti 8, an old cultivar. This likely explains its closer genetic affinity to the old cultivars rather than to more recently developed modern varieties.

Finally, the remaining modern cultivars (MC) clustered together (n=15), forming a distinct group (Figure 13, in teal green).

To further investigate the degree of genetic differentiation among the six identified populations, pairwise fixation index (F_{ST}) values were calculated (Figure 14).

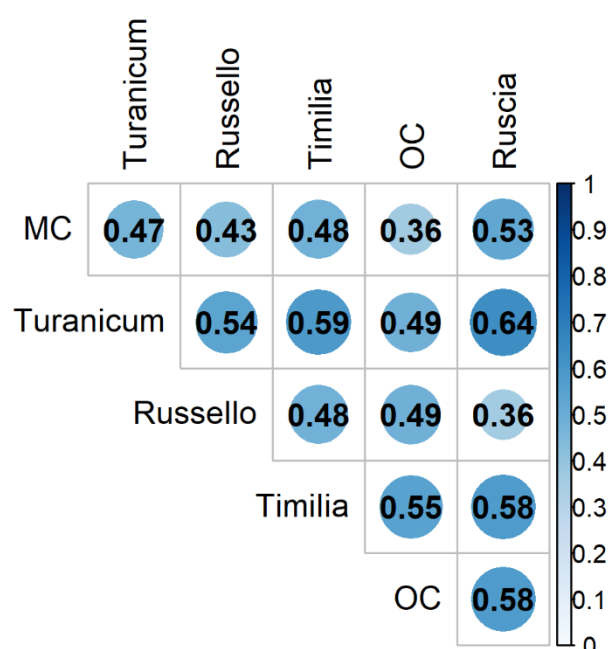


Figure 14 – Pairwise fixation index (F_{ST}) between the six population. OC = old cultivars, MC = modern cultivars.

The lowest levels of genetic differentiation were observed between OC and MC ($F_{ST}=0.36$) and between Ruscia and Russello ($F_{ST}=0.36$). In contrast the highest degree of differentiation was found between the *turanicum* and Ruscia populations ($F_{ST}=0.64$). Ruscia showed a $F_{ST}>0.50$ with all populations except Russello. *Turanicum* had highest F_{ST} with Ruscia, Russello and Timilia than OC and MC. MC and Russello showed tendentially lower fixation indexes.

6.3.3 Diversity analysis

A multidimensional scaling (MDS) analysis was performed on the dataset composed by 119 accessions and 1645 markers to visualize the genetic relationships and structure within the population (Figure 15).

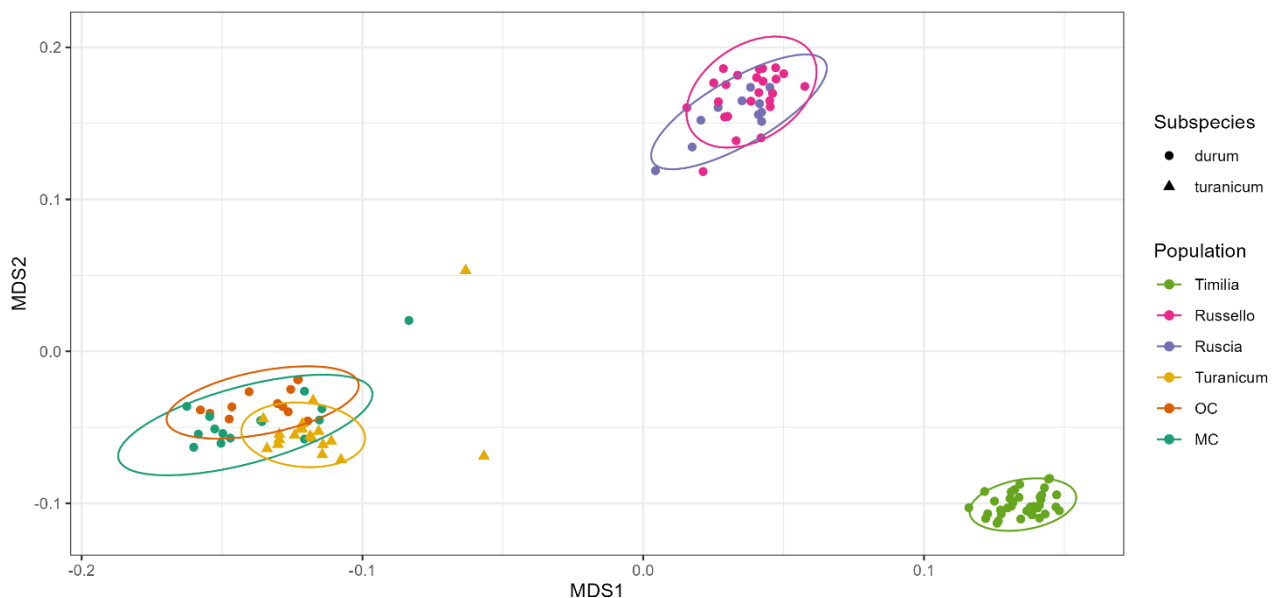


Figure 15 –Multidimensional scaling (MDS) plot of the 119 accessions with 1645 markers. Populations identified by PopCluster are marked with different colours. Different point shape indicates the subspecies. Ellipses represent the 95% confidence interval of each population.

The MDS analysis confirmed that the “Timilia” accessions formed a distinct cluster completely separated from all other populations, with no distinction between Timilia reste bianche and Timilia reste nere. The Russello and Ruscia populations were overlapped but remained distinct from the other populations. The clusters representing OC and MC were almost completely overlapped, probably due to the share of the same genetic background. Surprisingly, the *turanicum* cluster partially overlapped with OC and MC. Phylogenetic relationships among the accessions were furtherly studied constructing a neighbor-joining tree on Hamming distance. For improved readability,

Ruscia and Russello accessions share a common ancestral node, but they form two distinct clades.

All *T. turgidum* ssp. *turanicum* clustered together but no clear distinction among the accessions was found. In fact, Perciasacchi accessions did not cluster all together, indicating intra-variety variability. Notably some Etrusco, Saragolla lucana, Graziella Ra and Perciasacchi accessions resulted identical (Supplementary Table 1).

The MC and OC populations shared a common ancestral node in the phylogenetic tree. Within the OC population, two distinct sub-clades were identified. The first one included Bidì, Cappelli and Mahmoudi accessions, historical varieties derived from Tunisian landraces. The second one encompassed Capeiti 8 and Trinakria (which are descendent of Cappelli) and Simeto (derived from a cross involving Capeiti 8). It is also noteworthy that some Cappelli and Bidì accessions were found to be identical (Supplementary Table 1).

6.3.4 Statistics

To further study populations diversity, Nei's gene diversity (H_{exp}), Fixation index (F_{ST}) and the number of private alleles were calculated (Table 2).

Table 2 – Statistics of the different populations. N = number of individuals, H_{exp} = Nei's gene diversity (expected heterozygosity), F_{ST} = Fixation index. Private alleles are the alleles that occurs only in one population.

Population	N	H_{exp}	F_{ST}	Private alleles
MC	15	0.235	0.201	80
OC	12	0.152	0.476	11
Ruscia	11	0.112	0.614	5
Russello	24	0.201	0.319	45
Timilia	36	0.139	0.535	42
<i>Turanicum</i>	21	0.126	0.576	17

Nei's gene diversity (also called expected heterozygosity) measure the average genetic diversity per locus and is related to intra-population variability. Nei's gene diversity was

higher in MC ($H_{\text{exp}}=0.235$) and Russello ($H_{\text{exp}}=0.201$), followed by OC ($H_{\text{exp}}=0.152$), Timilia ($H_{\text{exp}}=0.139$), *turanicum* ($H_{\text{exp}}=0.126$) and Ruscia ($H_{\text{exp}}=0.112$) populations.

Fixation index (F_{ST}) gives information about the degree of differentiation of the populations. F_{ST} had an opposite trend in comparison with Nei's gene diversity, with values ranging from 0.201 to 0.614. MC had the major number of private alleles ($n=80$), followed by Russello ($n=45$) and Timilia ($n=42$). The lowest number of private alleles were found in *turanicum* ($n=17$), OC ($n=11$) and Ruscia ($n=5$).

An analysis of Molecular Variance (AMOVA) was conducted to assess the genetic structure of the populations and the results are reported in Table 3.

Table 3 – AMOVA results using 1645 markers of 119 accessions and considering 6 populations. Df = degree of freedom.

Source of variation	Df	Sum of squares	Percentage of variation	p-value
Between populations	5	55737.77	50.815	<0.001
Between samples within populations	113	59470.39	46.943	<0.001
Within samples	119	1461.07	2.243	
Total	237	116669.24	100	

AMOVA revealed that most of the variation in the analysed dataset was due to population structure accounting for 50.82%) of the total variance ($p\text{-value}<0.001$). A similar proportion of variation (46.94%, $p\text{-value} < 0.001$) was explained by differences between samples within populations. The remaining 2.24% of variation was attributed to sample variation, that represents the error of the model.

6.3.5 Traceability

Using a custom R script, a total of 18 markers were found able to discriminate the varieties Bidì ($n=5$), Ruscia ($n=2$) and Russello ($n=2$) and the two “populations” Timilia ($n=2$) and *turanicum* ($n=7$) (Supplementary Table 2). Unique alleles were found only for Timilia, while for the other varieties/populations, discrimination could be achieved with

the combination of markers. However, we were unable to find markers that could distinguish between *Timilia reste bianche* and *Timilia reste nere*, so these two varieties were treated as a single group. The same issue occurred with the *turanicum* accessions. Additionally, some *Bidì* accessions were identical to *Cappelli*, making it impossible to discriminate between these two varieties.

After identifying the 18 markers, we proceeded to design KASP (Kompetitive Allele-Specific PCR) assays to enable bi-allelic scoring, with the goal of achieving a rapid and cost-effective genotyping method. The assays were designed using the Polymarker software, which facilitates the development of homeoallele-specific markers (Supplementary Table 2). Their efficiency and specificity were empirically validated using a Bio-Rad CFX Duet Real-Time PCR system. All assays demonstrated clear allele discrimination among the tested samples.

6.4 Discussions

After having been abandoned for years, the cultivation of wheat conservation varieties is spreading again, due mainly to the interest of the consumers. Durum wheat is the most cultivated cereal in Italy, representing one of the most important crops economically and traditionally. Its history is tightly linked to this country, from the cultivation of landraces to the first breeding programs at the beginning of the 20th century. So, it is unsurprising that Italy has a richness in tetraploid wheat conservation varieties. They are particularly important in Sicily, where they are appreciated for their ability to be productive also in marginal areas.

For years, these varieties have been preserved in research institutes collections, but they have been mainly maintained and disseminated by farmers. This often led to varietal contamination, due to accidental seeds mixing pre- or post- harvest. Visioli et al. (2021), analysing glutenin composition, found degree of contamination ranging from 26% to 53% in conservation varieties grain batches provided by Sicilian farmers.

SNP microarray is a cost-effective and high-throughput technique to study genetic diversity in wheat. In the last year, many of them were developed and used for applications as genomic-wide association studies (GWAS), quantitative trait loci (QTL)

mapping, marker assisted selection (MAS) or population genetics studies (Esposito et al., 2024; Liu et al., 2024; Mazzucotelli et al., 2020; Sertse et al., 2023; P. Wang et al., 2022).

Several authors used SNP microarray to characterize wheat landraces and old cultivars (Ganugi et al., 2021; Mangini et al., 2018; Miazzi et al., 2022; Negisho et al., 2021; Taranto et al., 2020; Velimirović et al., 2023). Given the importance of wheat conservation varieties in Sicily, this technique was used to characterize them in several works (Fiore et al., 2019, 2022; Taranto et al., 2022).

In our work, we have collected numerous accessions of Sicilian tetraploid wheat conservation varieties directly from farmers responsible for their maintenance and commercialization with the aim to genetically characterize them using the Illumina Infinium 7K wheat microarray (Traitgenetics GmbH, Gatersleben, Germany). Together with these, we also collected other tetraploid wheat landraces, historical and modern varieties to use as a comparison. The final aim was to assess the diversity present today in these varieties and to develop a traceability method, useful for the valorisation of the supply chain.

The initial sample set was composed by 182 accessions, of which 141 Sicilian conservation varieties. First step of the analysis was the identification of duplicate or incorrectly identified samples. As stated above, it is common to find contaminated batch of seeds of wheat conservation varieties. Already during sample collection in the field, we found different spikes morphology, due both to natural variability, in the case of landraces, or to contamination. In the first case, spikes of each morphology type were collected; in the second case spikes were not collected. We used a multilocus genotype clustering and an identity-by-state (IBS) approach to identify and remove 45 duplicate and 17 misidentified accessions. Both approaches were based on Hamming distance calculation: the first was to find genetically identical individuals also in presence of missing data, the second was used to find accessions of a variety more similar to accessions of other varieties. Several instances of misidentification were identified during the analysis; in one case one accession of *Timilia reste bianche* was found to be identical to another of *Timilia reste bianche*. These two varieties derive from the same population “*Timilia*” and are mainly distinguished by the awn colour, yellowish in the first and black in the second (De Cillis, 1942). They are often confused, especially since

awn colour can be influenced by environmental factors. In the second case, seven accessions of *Timilia reste nere* and two of *Timilia reste bianche* were removed for their identity with Bidì and Cappelli accessions. Another *Timilia reste nere* accession (Timilia_rn_58B) was discarded because it clustered with Bidì and Cappelli accessions. Interestingly, these 10 accessions were sampled from two fields of two different farmers (Supplementary Table 1), highlighting the presence of contamination in different batches. In the third case, one Russello, two Ruscia, one Bidì and three Perciasacchi accessions were removed because falling outside the cluster of the relative varieties.

Most of the duplicated accessions identified in the analysis belonged to the same variety, with two exceptions. In one case, all Cappelli accessions were found to be identical not only to each other but also to four Bidì accessions.

Bidì and Cappelli are morphologically very similar and they are often confounded. The four Bidì accessions (Bidi_1A, Bidi_1B, Bidi_52A, Bidi_52B) were collected from two different farmers but exhibited an elongated spike, which is atypical for Bidì. This suggests that they have been misidentified. However, to retain valuable information, we decided to keep one of these accessions (Bidi_1B) based on its minimal missing data. In the second case, we found that fourteen Perciasacchi, four Etrusco, one Graziella Rà and one Saragolla lucana accessions were genetically identical. We decided to keep only one accessions for each variety, prioritizing those with the least missing data.

The final dataset composed of 119 accessions was used for genetic diversity analysis. The first step was the inferring of the population structure using a maximum likelihood clustering approach. An ideal number of six clusters was identified that represented the following populations: “*Timilia*”, “*Russello*”, “*Ruscia*”, “*turanicum*”, “*Old cultivars*” and “*Modern cultivars*”. The inferred populations were used for the subsequent analyses. AMOVA analysis validated this population structure. In fact, 50.82% of the variance found in our dataset was given by difference between populations, while 46.94% of the variance was given by differences between samples within populations, denoting also intra-population high variability. High pairwise F_{ST} index between populations (>0.36) further confirmed the calculated population structure.

The “Timilia” population contained 23 accessions of Timilia reste bianche and 13 accessions of Timilia reste nere. Neither multidimensional scaling plot nor neighbor-joining tree were able to discriminate between these two varieties. A relatively low Nei’s gene diversity index was found ($H_{exp}=0.139$), indicating low intra-population variability. This value was lower than the one ($H_{exp}=0.167$) found by Taranto et al. (2022) for the Timilia collection ($n=157$). This discrepancy is probably due to the lowest number of individuals present in our study. As stated before, these two varieties were selected from the same population on the basis of the awn colour. The results of our study confirm a strong genetic correlation between these two varieties. The impossibility to discriminate them could be attributed to the absence of appropriate markers in our dataset. Palombieri et al. (2024) found differences in ω -gliadins and low molecular weight glutenin subunits (LMW-GS) between the two varieties, suggesting genetic variation in the group 1 chromosomes. Interestingly, genes controlling awn colour are reported to be located in the same chromosomes group (Gadaleta et al., 2009). Admixture analysis indicated a very low degree of genetic admixture with other populations. Furthermore, the multidimensional scaling plot and the neighbor-joining tree highlighted a high genetic distance from other populations. Taranto et al. (2020) analysed 259 tetraploid wheat accessions using the Illumina iSelect 15K wheat SNP array, finding high distance between the nine Timilia accessions included in that study and the rest of the germplasm. Similar results were obtained by Fiore et al. (2022), that analysed 126 tetraploid wheats, including 14 Timilia accessions. All these findings collectively confirm the unique genetic origin of this landrace, suggesting that it has likely never been incorporated in durum wheat breeding programs. Given its distinct genetic profile and the potential insights it could offer, future studies should investigate its applicability and potential contributions to durum wheat breeding, especially in areas such as stress resistance, yield improvement, or quality traits.

Russello and Ruscia accessions formed two separate populations, with some degree of admixture between each other. Ruscia, also named “Russello ibleo”, is very similar to Russello, often leading to confusion between them. Neighbor-joining tree showed that these two varieties derived from the same ancestral node. Moreover, multidimensional scaling analysis revealed an almost complete overlapping of the two varieties clusters.

Pairwise F_{ST} index between the two populations was equal to 0.36, one of the lowest found in this study. Russello population was characterized by a higher Nei's gene diversity index (0.201 vs 0.112 of Ruscia), a lower F_{ST} index (0.319 vs 0.614 of Ruscia) and a higher number of private alleles (45 vs 5 of Ruscia). These data highlight that Russello is characterized by higher intra-population variability than Ruscia.

All accessions of *T. turgidum* ssp. *turanicum* clustered together, highlighting their genetic similarity. Interestingly, all Perciasacchi accessions did not clustered separately from the other *turanicum* varieties. In the neighbor-joining tree two different clusters emerged: one containing only Perciasacchi accessions and one containing some Perciasacchi accessions alongside those from the other *turanicum* wheat. Additionally, two Perciasacchi accessions exhibited unique clustering behaviour, as evidenced by their separation in both the neighbor-joining tree and the multidimensional scaling plot. This suggests potential genetic diversity within the Perciasacchi accessions, warranting further investigation into their genetic relationships and distinctions within the *turanicum* subspecies. In this, Perciasacchi cluster overlap with OC and MC clusters. In our work, the *turanicum* accessions showed more similarity with durum wheat old cultivar and modern cultivar than with Sicilian landraces. This result is in accordance with Fiore et al. (2022), where four *turanicum* accessions, including two Perciasacchi (called by the synonym Farro lungo), shared the same ancestral node with old and modern cultivars. As said above, we found identity between fourteen Perciasacchi, four Etrusco, one Graziella Ra and one Saragolla lucana accessions. All these *turanicum* varieties originate from Italy, probably sharing the same genetic background. At the moment, we cannot determine whether the elevated identity among these varieties is due to actual homonymy, misidentification, or the lack of adequate markers for discriminating the *turanicum* varieties. It is important to consider that wheat microarray platforms and SNP dataset, such as those used in this study, were developed based mainly on *T. aestivum* and only marginally on *T. turgidum* ssp. *durum* (S. Wang et al., 2014; M. O. Winfield et al., 2016).

OC cluster was composed by Bidì, Capeiti 8, Cappelli, Mahmoudi, Trinakria and Simeto accessions. Mahmoudi is a Tunisian landrace and Bidì and Cappelli are old cultivars selected at the beginning of the 1900 from Tunisian landraces. Neighbor-joining tree

showed that they share the same ancestral node. It is commonly that Cappelli was selected by a Tunisian landrace called Jenah Retifah, while Bidì was selected from Mahmoudi. However, De Cillis (1927) reported that Bidì and Cappelli were selected from the same Tunisian landrace. Recent studies seem to support this hypothesis and also our data shows the genetic similarity between Mahmoudi, Bidì and Cappelli (Fiore et al., 2022; Marzario et al., 2018; Taranto et al., 2020). Capeiti 8, Trinakria and Simeto are cultivars developed within Italian breeding programs and share a common genetic origin through the historical variety Cappelli. In fact, Capeiti 8, released in 1955, originates from a cross between Cappelli and Eiti 6. Trinakria, released in 1970, derives from a cross between Capeiti 8 and B14. Finally, Simeto derives from a cross between Capeiti 8 and Valnova and was released in 1988. These genealogical relationships explain the genetic similarity observed among these cultivars. Consistently, the “old cultivars” (OC) and “modern cultivars” (MC) clusters showed a low level of genetic differentiation, as indicated by a pairwise F_{ST} value of 0.36, the lowest observed among the analysed groups. Furthermore, these two clusters largely overlapped in the multidimensional scaling (MDS) analysis, further supporting their shared genetic background.

Last cluster (MC) contained all the modern cultivars included in this study. These are all varieties released in the 21st century and they are still cultivated in Italy. This cluster showed the highest Nei's gene diversity index ($H_{exp}=0.235$) and number of private alleles ($n=80$), probably due to its high heterogeneity. The fixation index was the lowest ($F_{ST}=0.201$), indicating less differentiation compared to the other populations.

Summing up, we analysed the diversity of Sicilian tetraploid wheat conservation varieties, finding high inter- and intra- populations variability. Notably, high variability and genetic distance were found between Sicilian landraces and modern cultivars, especially in the case of Timilia, highlighting the opportunity to include them in future breeding programs. Our findings complement and expand upon the previous study by Taranto et al. (2022) and Fiore et al. (2022), providing deeper insights into the phylogenetic relationships among Sicilian conservation varieties and reinforcing their relevance for conservation and pre-breeding efforts. Moreover, we investigated the genetic relationship between Italian *turanicum* accessions, obtaining unexpected results. Specifically, we found genetic complete identity among accessions of Etrusco,

Graziella Ra, some Saragolla lucana and some Perciasacchi samples. These varieties are also morphologically indistinguishable, which complicates the assessment of whether the observed identity is due to actual synonymy, misidentification, or a lack of discriminating markers. The cultivar QK-77 also showed high genetic similarity to other *turanicum* accessions studied. Overall, our findings suggest a shared genetic origin among these varieties and highlight the need for further studies to investigate the global genetic relationships within the *turanicum* subspecies.

Another objective of our work was the development of a DNA-based traceability system for Sicilian tetraploid wheat conservation varieties. KASP assay is a simple and convenient way to perform allelic discrimination, that can be applied to all plant species (Carrara et al., 2023; Sales et al., 2023; W. Tang et al., 2022; M. Winfield et al., 2020). We decided to select the minimum number of SNPs that were able to distinguish among the varieties Bidì, Russello and Ruscia and the accessions belonging to Timilia population and *turanicum* subspecies. Using a bioinformatic approach, we found a total of 18 SNP markers suitable for this purpose, which were subsequently converted into KASP assays. As highlighted by our findings, misidentification is recurring issue within the conservation varieties supply chain. From a consumer standpoint, ensuring the authenticity of raw materials is essential, especially when products command a premium price due to their traditional or geographical origin. The set of markers we developed can be applied across different points of the supply chain to prevent fraud, support traceability, and enhance product value.

6.5 Supplementary materials

Supplementary materials can be found at the following link:
[https://drive.google.com/drive/folders/1f-B9YFb9MJrqpbmPsGMOqOMOivlw6r39?usp=drive link](https://drive.google.com/drive/folders/1f-B9YFb9MJrqpbmPsGMOqOMOivlw6r39?usp=drive_link)

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7 Conclusions

In this work different accession of durum wheat relatives and Sicilian tetraploid conservation varieties were analysed using biochemical and genetical approaches.

The rising importance of these varieties makes it necessary to evaluate their characteristics to valorise them providing validated information to consumers. Moreover, genetic analyses are essential for their conservation and traceability. Despite their poor yield and technological quality, their ability to grow in marginal areas and their resistance to abiotic stresses makes them a valuable resource for farmers and breeders.

Our work on Polish and Khorasan wheat demonstrated that most of the variability in mineral and fiber content and root morphology was due to genotype and not to subspecies. These results highlight the importance of focusing on the genotype and its interaction with the environment in comparative studies, and not generically on subspecies. Despite their poor rheological parameters, Polish and Khorasan wheat showed pasta making properties comparable to the modern cultivar Svevo.

This study provided an in-depth analysis of the conservation varieties. Both genetic and gluten protein composition analyses revealed great intra-varietal variability present in Sicilian landraces. Two different approaches for varietal traceability in the supply chain were developed: one based on gliadin and glutenin subunits electrophoretic patterns and one based on SNPs analysis with KASP markers. In a multi-environment trial, we evaluated the ATIs and fructan accumulation of five Sicilian conservation varieties and three modern durum wheat varieties. We did not find differences in total ATI content between conservation varieties and modern cultivars, but only slight differences in minor ATI composition. Tendentially, conservation varieties accumulated less fructans, especially in the cases of Bidì and Russello. Environmental influence was significative in the accumulation of both compounds, but high heritability was found except for total ATI. Finally, our studies revealed that Bidì and Russello, under certain environmental conditions, can show similar %UPP of modern cultivars.

In conclusion, tetraploid wheat subspecies and conservation varieties have peculiar characteristics that can be exploited in modern breeding. This work gives data and

methodologies to support and valorise their supply chain and improve the knowledge on the differences between old and modern genotypes, shedding light on the causes of their putative healthier properties, if such benefits are substantiated.