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Proceeding

Nutritional and genetic assessment of Traditional Greek Wheat Varieties.†

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Abstract: Research has highlighted the nutritional benefits of ancient grains, especially emmer (*Triticum dicoccum*) and einkorn (*Triticum monococcum*), compared to modern wheat varieties, focusing on their higher levels of antioxidants and phytochemicals. The samples studied in this research included emmer and einkorn as well as common wheat and durum wheat grains. The Internal Transcribed Spacer 2 (ITS2) nuclear region was amplified and sequenced as a barcode for species identification, allowing einkorn discrimination. The total content polyphenols and flavonoids, as well as the antioxidant activity of emmer and einkorn were higher than common and durum wheat varieties.

Keywords: Wheat varieties; biochemical analysis; phytochemicals; plant barcoding

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1. Introduction

Wheat (*Triticum aestivum L.* ssp. *aestivum*, or 'common' wheat and *Triticum turgidum L.* ssp. *durum*, or 'durum' wheat) is the third most cultivated crop in the world, after maize and rice [1]. It covers the largest surface among cereals, 730 million hectares worldwide in 2018, with a total yield of 2810 million tons in 2021 [2]. The most common commercially available old wheat species are einkorn (*Triticum monococcum* ssp. *monococcum*), emmer (*T. turgidum* ssp. *dicoccum*) also known as farro in Italy, khorasan (*T. turgidum* ssp. *turanicum*) and spelt or dinkel (*T. aestivum* ssp. *spelta*) [3,4]. In Greece, at the beginning of the 20th century, only one cultivated variety of einkorn is found, while there are no references to the use and cultivation of emmer wheat and spelt in the Greek territory for at least the last 150 years [5,6]. However, only a very few local producers are making efforts to reintroduce these varieties. In a balanced diet, whole-grain wheat is a healthy source of nutrients, fiber, and bioactive compounds. Given the growing concern over diet-related chronic diseases and increased mortality worldwide, there is significant interest in improving wheat for better health [3]. Ancient wheat varieties, in particular, have gained attention because of their capacity to perform in poor soils and low irrigation and for potentially offering a more nutritious profile compared to modern wheat, with higher vitamin, mineral, and nutraceutical content [7]

The aim of this study was to use the Internal Transcribed Spacer 2 (ITS2) nuclear region as a barcode for species identification and to evaluate nutritional factors of emmer and einkorn as well as common wheat and durum wheat grains, like polyphenols, flavonoids, antioxidant activity and fatty acids composition.

2. Materials and Methods

2.1. Samples

The material selected for the study comprised grains of four groups of wheat varieties, i.e. emmer, einkorn, common wheat and durum wheat. Table 1 shows the categorization of the selected samples. All samples were obtained from a certified producer in Greece (Antonopoulos farm, Dilofo). All plants were grown in the same location and within the same year in order to minimize differences in their environmental conditions.

Table 1. Samples of wheat varieties used in the study.

Common Wheat	Durum Wheat	Emmer	Einkorn
Oropos	Skliro_Deveta_Dilofou	Emmer_Greece	Einkorn_Greece
Malako_asprositaro	Palaio_skliro	Emmer_Italy	Einkorn_Italy
Malako_oreinou_Dilofou	Limnos		
Generoso	Mexikali 81		
Yecora	Simeto		

2.2. DNA barcoding

DNA was extracted from 200 mg seeds of each sample using the kit NucleoSpin Food, Macherey- Nagel (Dueren, Germany), according to manufacturer's instructions. For species identification the Internal Transcribed Spacer 2 (ITS2) region of each sample was PCR amplified using the primers ITS2_F (ATGCGATACTTGGTGTGAAT) and ITS2_R (GACGCTTCTCCAGACTACAAT) [8]. Each PCR reaction consisted of 100 ng genomic DNA, 1 unit of Xpert Fast DNA polymerase (Grisp, Porto, Portugal), 5×Xpert Fast Reaction Buffer and 0.4 μL of each 10 μM ITS2 primer, at a final volume of 20 μL. The PCR reactions were performed at: 95˚C for 1 min, then 40 cycles of 95˚C for 15 s, 15 s at 45˚C, 72˚C for 3 s, and one final extension step at 72˚C for 3 min on a Thermocycler (Thermo Fisher Scientific, Waltham, MA, USA). Successful PCR reactions were purified with the PureLink™ PCR Purification Kit (Thermo Fisher Scientific, Waltham, MA, USA), and then used for Sanger sequencing with ITS2_F primer, on the ABI3730xl platform (Cemia, SA, Larissa, Greece). Sequence alignment was performed using the Muscle feature of MEGA- X software (https://www.megasoftware.net/) [9]. The sequence identity was found by applying Nucleotide Blast feature of NCBI. MEGA- X software was again used to construct a Neighbor-Joining phylogenetic tree with reference sequences for each species retrieved from NCBI.

2.3. Extraction of Free and Bound Phenolic Compounds

The free and bound phenolic compounds were extracted according to Kaur et al., 2021 with minor modifications [10]. Briefly, for free phenolics, 0.5 g of grounded wheat grains were homogenized with 5 ml of 80% MeOH and sonicated in an ultrasonic water bath. After centrifugation at 4000 rpm for 10 min, the supernatant was removed and the residue was resuspended in 80% MeOH (5 ml) and extraction was repeated twice. Supernatants were pooled and dried at 45˚C using rotavapor under reduced pressure. The residue left was further digested with 10 ml of NaOH (2 M) at room temperature for 2 h for the extraction of bound phenolic compounds. The pH was adjusted to 2 and the mixture was centrifuged at 4000 rpm for 5 min. The supernatant was extracted with 10 ml of ethyl acetate/diethyl ether solution (1:1 v/v) three times. The organic layer was collected and evaporated to dryness. Both free and bound phenolics dry residue were re-dissolved in 2 ml of 80% MeOH, filtered through a 0.45 μm filter. The extracts were used for the determination of free and bound phenolics, flavonoids, as well as to assess antioxidant activity.

2.4. Total phenolic content (TPC)

Total phenolic content of each extract was estimated spectrophotometrically using a modified Folin-Ciocalteu colorimetric method [11,12]. Briefly, 50 μl of the appropriate diluted extracts were mixed with 600 μl of distilled water followed by the addition of 50 μl Folin-Chiocalteu reagent. The samples were mixed well and allowed to stand for 8 min.

The reaction was neutralized by adding 300 µl of 20% Na_2CO3 and the absorbance of the solution was recorded at 760 nm on UV-Vis spectrophotometer (Shimadzu UV-2600, Kyoto, Japan) after 60 min. The free, bound and total phenolics were reported as mg gallic acid equivalent (GAE) / 100 g of grain.

2.5. Total flavonoid content (TFC)

The TFC of free and bound extracts were quantified using a colorimetric method described previously by Liu et al. (2002) [13] with some modifications. Dilutions of sample extracts reacted with $NaNO_2$ (5%), followed by reaction with $AlCl_3 \cdot H_2O$ solution (10%) to form a flavonoid-aluminum complex. Solution absorbance at 510 nm was immediately measured using a UV-VIS spectrophotometer. The free, bound and total flavonoids were expressed as mg quercetin (QE)/100 g of grain.

2.6. Determination of antioxidant activity

The ability of the extracts to react with radical of 2,2′-diphenyl-1-picrylhydrazyl (DPPH) and was used to determine their antioxidant activity [14]. Briefly, 25 µl of free and bound extracts reacted with 975 µl freshly prepared DPPH solution (6×10^{-9} mol l^{-1}) and incubated at room temperature for 30 min. The absorbance of the solution was measured at 515 nm using a UV-VIS spectrophotometer. Results were expressed as the percentage of DPPH neutralization.

2.7. Analysis of fatty acids methyl esters (FAMEs)

Fatty acids were extracted and determined according to the direct FAME synthesis method described by O'Fallon et. al (2007) [15]. Fatty acids composition was determined using a GCMS-QP2010 Ultra Gas chromatograph mass spectrometer (Shimadzu Europe, GmbH) equipped with a SP-2340 capillary column (60 m × 0.25 mm, 0.20 µm film thickness) (Supelco, Bellefonte, PA, U.S.A.). The injector and flame ionization detector were both set to a temperature of 250°C. Initially, the oven temperature was set at 100°C for 5 minutes and then gradually increased to 240°C at a rate of 4°C per minute, holding at this temperature for 30 minutes. Helium was used as the carrier gas for the analysis at a flow rate of 20 cm/min. For identification and calibration purposes, the Supelco 37 Component FAME Mix (Sigma-Aldrich) was employed. The composition of fatty acid methyl esters (FAMEs) was expressed as the relative percentage of each fatty acid, calculated through internal normalization of the chromatographic peak areas.

2.8. Statistical analysis

For each biochemical analysis, triplicate measurements were conducted, and data were expressed as mean value ± standard deviation (n=3). Statistical analysis was performed using t-test (GraphPad, San Diego, CA, USA), while p-value significant threshold was 0.05 ($p \leq 0.05$).

3. Results

ITS2 sequencing results revealed that the einkorn samples shared the exact same sequence, while all the other samples of wheat and emmer shared a different one, as attended, with minor sequence differences. The phylogenetic tree of the ITS2 sequencing results (Figure 1) showed the formation of two branches, one with the einkorn samples and one with all the wheat and emmer samples.

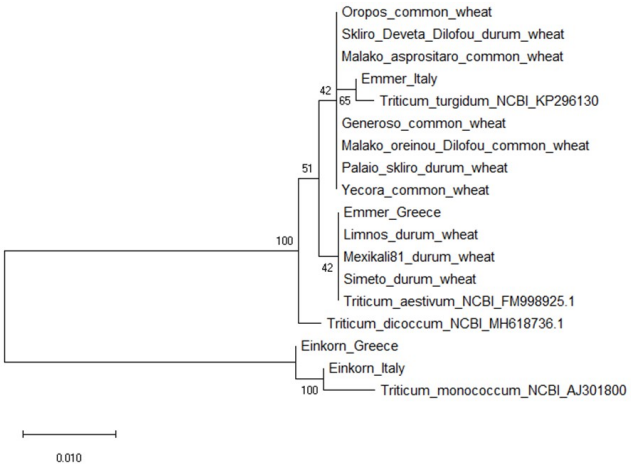
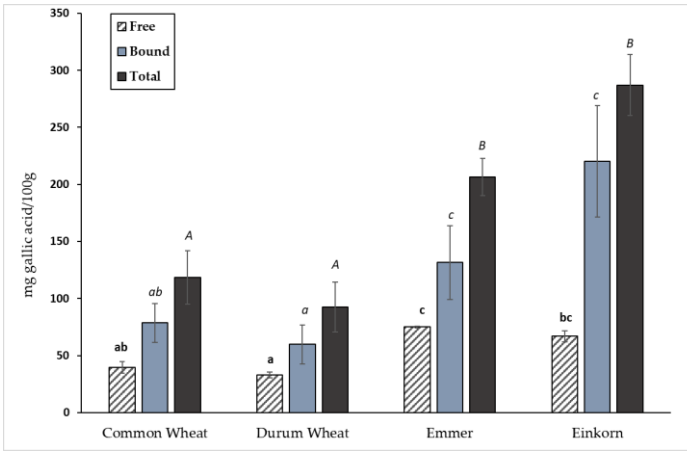
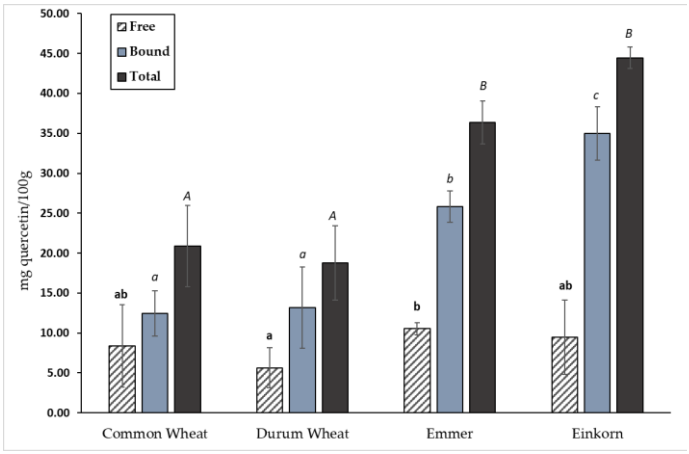


Figure 1. Phylogenetic tree of all the samples sequenced, using Neighbor-Joining method.

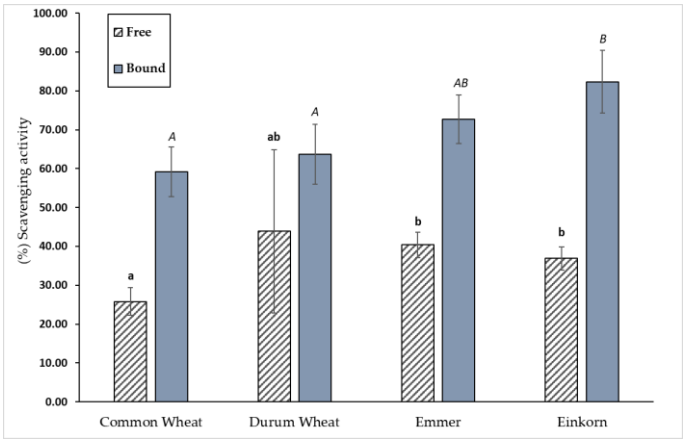
Free and bound phenolic contents of emmer and einkorn were significantly higher than those of common and durum wheat (Figure 2a). Total phenolic content was highest in einkorn, and this level was significantly different from those in common and durum wheat, but was similar to levels in emmer. Free flavonoid content of wheat varieties ranged from 5.63 ± 2.51 mg QE/ 100 g in durum wheat to 10.53 ± 0.75 mg QE/ 100 g of grain in emmer (Figure 2b). The free flavonoid content of emmer and einkorn was similar to common wheat. Total flavonoid content was lowest in durum wheat and highest in einkorn. Free scavenging activity of einkorn, emmer and durum wheat were not statistically different (Figure 2c). Einkorn exhibited significantly greater bound total scavenging capacity than common and durum wheat.



(**a**)



(**b**)



(**c**)

Figure 2. (**a**) Concentration of free, bound and total phenolic compounds of wheat varieties expressed as gallic acid equivalents per 100g.; (**b**) Concentration of free, bound and total flavonoid compounds of wheat varieties expressed as quercetin equivalents per 100g. (**c**) Scavenging activity (%) of free and bound extracts of wheat varieties. Different letters indicate statistically important difference ($p \leq 0.05$).

In all samples, five primary fatty acids were identified, listed in descending order of their respective quantities: linoleic acid (C18:2) > palmitic acid (C16:0) ≈ oleic acid (C18:1) > eicosenoic acid (C20:1, cis-11) > stearic acid (C18:0) (data not shown). Percentage (%) content of mono-unsaturated fatty acids (MUFA) is shown in Figure 3. Emmer and einkorn had significantly higher % MUFA than common and durum wheat. Common wheat had the lowest % of MUFA between all varieties.

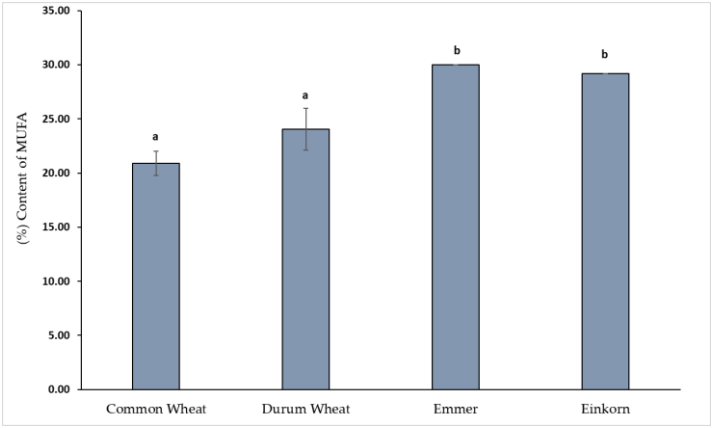


Figure 3. Presentence content of mono-unsaturated fatty acids (MUFA) of wheat varieties.; Different letters indicate statistically important difference ($p \leq 0.05$).

3. Discussion

The nuclear ribosomal DNA- internal transcribed spacer 2 (ITS2) has been recognized as a suitable genomic region for elucidating genomic and phylogenetic relationships among plants [16]. When used to wheat samples it has been shown that it has poor discrimination power between *Triticum* species [17,18]. That was confirmed by our results as well, since ITS2 did not discriminate *T. turgidum* (that includes emmer and durum wheat) from *T. aestivum* samples. However, einkorn samples (*T. monococcum*) were clearly discriminated using this marker.

Wheat (*Triticum* spp.) is consumed by billions of individuals and serves as the primary staple food in numerous diets, contributing significantly to the daily energy intake for many people. The consumption of grains has been linked to a lowered risk of specific long-term health conditions, and this has been attributed in part to the unique phytochemicals in grains [3]. Available data indicates that ancient wheat cultivars tend to have lower levels of certain components, such as dietary fiber, while being higher or distinctive in other components, such as polyphenols. [5].

Our findings highlighted variation between wheat species, with einkorn exhibiting the highest phenolic content, which is in accordance with other studies, reporting that einkorn exhibited higher content of both free and bound polyphenols than common and durum wheat [19,20]. Laus and colleagues assessed the antioxidant activity across a wide range of Italian ancient and modern wheat varieties, and they concluded that there were negligible variations, asserting that the potential of modern varieties had not diminished despite a century of breeding efforts [21]. However, our results suggest a higher phenolics and flavonoids content in ancient wheat.

The comparative analysis of fatty acid composition in wheat varieties, einkorn revealed 53% higher proportion of monounsaturated fatty acids than common wheat according to Hidalgo et. al (2009) [22]. Into our study it was proved that einkorn and emmer had significantly higher proportion of MUFA than common and durum wheat.

The results of this study demonstrate the high nutritional value of emmer and einkorn to common wheat, underlining the importance of maintaining sustainable agricultural practices to ensure their continued cultivation.

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