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# MODERN METHODS IN CEREALS AUTHENTICATION

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***Abstract***

*Common and durum wheat are widelly used for many purposes and the prices are significantly different for each wheat type, thus efficient methods for detection of accidental or intentional admixtures of common wheat to durum wheat products, and vice-versa, are required. The aim of this study is to identify some characteristics of the Triticum aestivum genetic material witch will be useful in developing methods for determination of the quality and authenticity of this species. Melting, DNA methylation and RAPD analysis can be used to discriminate between wheat species/varieties.*

***Key words****: Triticum aestivum, authentication, food safety, Romania.*

## INTRODUCTION

Wheat is the world's most prevalent plant due to various industrial use.
Romania has approximately 10 million hectares of cultivated arable land (more than 2 million hectares are cultivated with wheat). Main production area is the Danube plain in the south of the country. Other important wheat growing areas are Transylvania, the northern part of Moldova in north-eastern Romania and the Banat region in the South West. According to data preserved by Ministry of Agriculture and Rural Development, wheat has the largest acreage in Romania (2043.0 thousand hectares in 2015), recording a continuously growing production (in 2015 3842 kg/ha) (http://www.madr.ro/culturi-de-camp/cereale/grau.html).
At present, a large number of wheat varieties were obtained by genomic selection or genetic engineering (Bhalla et al., 2006).
From those, *Triticum aestivum* L. (also named *Triticum vulgare*, *Triticum aestivum* subp. *aestivum*) and *Triticum turgidum* ssp. *durum* (includes durum and red durum) are the most cultivated species of wheat. *Triticum aestivum* L. (common wheat) is a hexaploid species of wheat.
(www.ogtr.gov.au/internet/ogtr/publishing.nsf/Content/wheat-3/$FILE/biologywheat08.pdf).
It is almost completely processed into flour and the rest is used in different types of food.
*Triticum turgidum* ssp. *durum* is mainly used for the production of pasta and macaroni, but as well for the production of bread.
Determination of the quality and authenticity of *Triticum* wheat is important for industry, food safety and consumer protection (Knodler et al., 2010). The investigation field is broad (Korzun et al., 1997; Pegels et al., 2015; Prandi et al., 2012) due to growing wheat variety, the large number of industrial processing steps and also to merchandising diversity of bakery and pastry products.
Several methods that can identify varieties that are genuine from forged products (substitution of wheat varieties with other grains species of wheat or mixtures in different proportions) are described in the literature (Arlorio et al., 2003). Some of these methods are based on molecular biology techniques (e.g. particularly the analysis of specific protein fractions and DNA analysis of wheat varieties) (Osborne, 1996). Peculiarities of wheat varieties, degree of maturation, ecological conditions, soil processing techniques, physico-chemical

composition and technological peculiarities make the standardization of these methods and identification of wheat species/varieties even more difficult.
The aim of this study was to identify some characteristics of the *Triticum aestivum* L. genetic material which will be useful for developing methods for determination of the quality and authenticity of wheat species.

## MATERIALS AND METHODS

The analysed wheat samples were: *Triticum aestivum* Glosa variety, *Triticum aestivum* Excelsior variety, *Triticum durum* Condurum variety, *Triticum durum* Grandur variety, *Triticale* Haiduc variety (all of them are originated from National Agricultural Research and Development Institute Fundulea experimental plots, Calarasi county). The samples were collected in 2014. Each sample consisted of approximately 500 g of wheat. The samples were selected after harvesting.
The characteristics of these samples were analysed by molecular methods based on the Random Amplified Polymorphic DNA (RAPD) technique, thermal denaturation profile of DNA molecules and DNA methylation analysis.
DNA was extracted from the selected probes with SureFood® PREP Plant X (R-Biopharm, Germany) according to manufacturer's instructions. The quality of the DNA extract was verified by electrophoretic and spectrophotometric methods (Beckman DU170 spectrophotometer). Every DNA sample was considered as an average of two measurements and it was accepted if the acceptance range for DNA quality value was between 1.7 and 2.

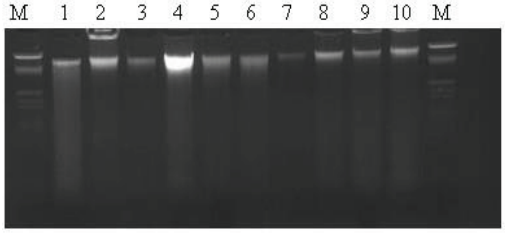


Figure 1. Agarose gel electrophoresis from wheat genomic DNA. Lines: 1,2-*Triticum aestivum* Glosa variety; 3,4-*Tritcum aestivum* Excelsior variety; 5,6-*Triticum durum* Condurum variety; 7,8-*Triticum durum* Grandur variety; 9,10-*Triticale*; M marker Lambda DNA/EcoRI + Hind III

*1. Analysis of thermic denaturation curves in the presence of fluorophore- Melting Analysis*
Initially an incubation of mixture at 35ºC/2 minutes was used to settle the basal fluorescence of samples. Denaturation curve of DNA samples was recorded, based on a series of fluorescence readings every minute, with a 5 seconds incubation at constant temperature before every reading (to allow fluorescence stabilization of the DNA denaturation equilibrium at every step).
Denaturation curves and data analysis were performed with HRM or melting analysis on a Corbett RotorGene 6000 (HRM module) device.
*2. DNA Methylation analysis*
The methylation profiles of DNA were analysed with izoschizomere enzymes Msp and Hpa. Reaction mixture contained: DNA (20 µl), restriction endonuclease (10 U), restriction buffer (2.5µl), $H_2O$dd till final volume of 30µl. Reaction mixture was incubated for 3 hours at 37ºC, in continuous stirring water bath. Restriction reaction was stopped by adding loading buffer. The restriction products were resolved by electrophoresis (agarose gel 0.8%).
*3. RAPD analysis*
Hexanucleotide OPA primers were used for RAPD technique. The composition of the mixture used for amplification was: PCR buffer 1.2 µl, dNTP 0.4 µl, Taq polimerase 0.4 µl, OPA primers 0.25 µl, double-distilled $H_2O$ 8.75 µl, DNA 1 µl. The amplification products were separated in 2% agarose gel (stained with ethidium bromide whereas the small fragments were separated in PAGE 8%).

## RESULTS AND DISCUSSIONS

*1. Profile analysis of thermal distortion in the presence of fluorophore - Melting Analysis*
Denaturation curves analysis can be used to characterize the genome composition in nitrogenous bases and for DNA molecules discrimination. An improvement of the technique was made by introducing fluorescent dyes. These methods allow the accurate recording of fluorescence changes at temperature variations of at least 0.1°C.
The denaturation curves profile for all DNA samples were heterogeneous and, regardless of

fluorophore used (e.g. SYBR Green or Eva Green), the results of analysis were difficult to interpret. One possible explanation of these results was the different distribution of AT or GC base pairs in different regions of genome of the analysed species. The optimization method was performed by changing the amount of DNA (10-25 µl), the range of temperature used for denaturation (55-98ºC), the frequency of fluorescence readings (1 fluorescence reading at every 0.1ºC, 0.5ºC or 1ºC sample warming) and the fluorophore used.

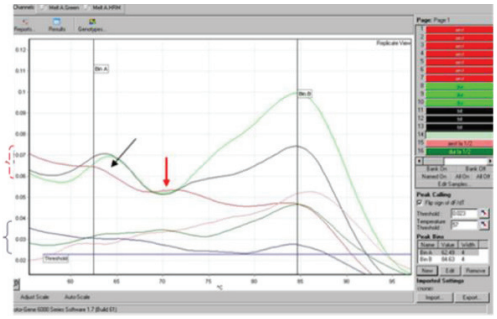

Figure 2. Denaturation profiles for *Triticum durum* Condurum variety; *Triticale* Haiduc variety and *Triticum aestivum* Glosa variety (melting analysis)

In Figure 2 the upper curve was obtained from an amount of 2.5 times higher than bottom one (SYBR Green 1x; denaturation range 55 to 95ºC, 1 reading at 0.5ºC, replicate view).

The effect of the amount of DNA used for each determination is revealed in the Figure 2. Our results indicated that at low concentrations of DNA certain peaks in the distortion curve are difficult to be identified. Thus, our results regarding the effect of the amount of DNA on the denaturation curves are consistent with previously published data in other scientifical papers. The best results were obtained for reading of 25 µl DNA, recording of fluorescence with a frequency of 1 reading at every 1°C increase of sample temperature, with a heavy digital filter for fluorescence noise and in replication analysis module.

Repeating the experiment showed similar patterns of bands, with similar differences (replicated view).

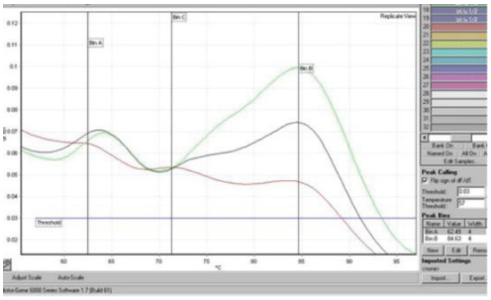

Figure 3. Analysis of melting curves showed some variation features of fluorescence between the analysed species of wheat. The most evident differences were recorded when the fluorescence was read at every 1°C increase of sample temperature (replication results, heavy digital filter). *Legend:* red – denaturation curve for *Triticum aestivum*, green- denaturation curve for *Triticum durum,* black -denaturation curve for *Triticale*

Melting analysis may reveal significant differences between *Triticum aestivum*, *Triticum durum* and *Triticale*. The differences between melting curves obtained for *Triticum aestivum*, *Triticum durum* and *Triticale* was most evident for 60-68°C and 83-92°C temperature interval. These differences may reflect the percent of AT and GC in these region. In the range of 60-68°C the pattern of denaturation curves obtained for *Triticum durum* and *Triticale* samples differs significantly from that produced by *Triticum aestivum*. These regions may be used in development of molecular methods useful for identification of species of wheat.

*2. DNA Methylation analysis*

DNA methylation occurs in both animals and plants. The first applied model was the plant *Arabidopsis thaliana* (Cokus et al., 2008) and, since then, has been applied to many plant species. Genomic DNA methylation in plants can occur symmetrical or non-symmetrical. Investigation of DNA methylation may provide new data about factors which may involve in gene transcription and phenotypic variation in booth plants and animals. Our results are in agreement with the data reported in the literature (Gardiner et al., 2015).

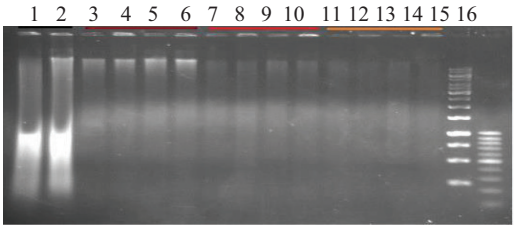


Figure 4. Agarose gel electrophoresis from wheat genomic DNA.
Legend: Lines: 1-2 DNA mammal verification; 3-6 Triticale; 7-8: *Triticum durum* Grandur variety; 9-10: *Triticum durum* Condurum variety; 11-12: *Triticum aestivum* Excelsior variety; 13-14: *Triticum aestivum* Glosa variety; 15: 1kb GeneRuler DNA Leadde (Fermentas); 16: 100 bp GeneRuler DNA Leadde (Fermentas), (0.8% agarose gel).

*3. RAPD analysis*

Random Amplified Polymorphic DNA analysis is a technique which does not require any specific knowledge of the DNA sequence of the target organism. RAPD analysis showed that a low number of genetic markers for drought tolerance in wheat are polymorphic (18.6%) (Deshmukh et al., 2012).

We determined 4-12 electrophoretic bands/ sample of DNA, according to the primers used and the concentration of the genomic DNA extract from *Triticum aestivum* Glossa variety and *Triticum durum* Condurum variety. Most of the bands resulted were not polymorphic (polymorphic bands are indicated in the brace and the arrows indicate monomorphic bands). However, the bands number and pattern is insufficient to determine efficiently the wheat species.

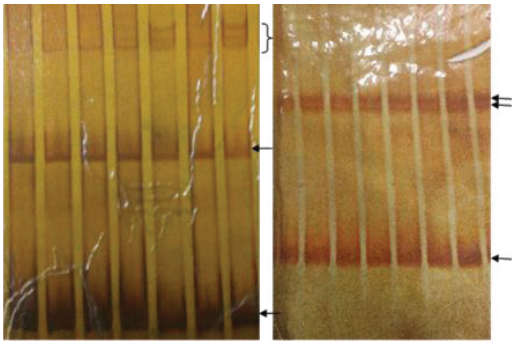


Figure 5. The image of an acrylamide gel, where there were separated amplification products with different random hexamer primers type OPA (A, B)

## CONCLUSIONS

Based on the results obtained in this study we can formulate the following conclusions:

Melting analysis may reveal differences between *Triticum aestivum*, *Triticum durum* and *Triticale*.

The mehtylation profile presented the biggest differences in *Triticale* comparativ to *Triticum aestivum* and *Triticum durum,* and the smallest differences in *Triticum aestivum* and *Triticum durum.* The mehtylation profile was similar within the samples from the same species.

The RAPD analysis showed the existence of 4-12 bands that can be used to determine wheat species. The results can be influenced by the amount and purity of the genetic material used in the reaction.

The varieties/species of wheat can also be determined with the help of molecular analysis, but they need some special conditions, related to the amount and the quality of the genetic material needed for analysis.

## ACKNOWLEDGEMENTS

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