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Genomic analysis of commercial varieties of tetraploid wheat

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Abstract

Wheat is one of the most important crops in the world and just last year the crop area of durum wheat in Portugal suffered a reduction of 69%, this way it becomes more important to characterize the *Triticum durum* commercial varieties available for crop production. In this work we used six durum wheat varieties usually planted in Portugal (Celta, Hélvio, Marialva, Don Duro, Saragolla and Severo), with the purpose of studying their genomic variability, using PCR-based molecular techniques.

Our results indicate that, although being reproduced by self-fertilization, durum wheat's varieties still show some genomic variability, not only among different varieties, but also between individuals from the same variety. We have also showed that, even though being commercial varieties, not all of them appear to have dough with superior end use quality.

This variability can help us develop specific primers to easily distinguish different varieties, and aid in the identification and characterization of varieties with better quality traits.

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1. Introduction

Together with rice and maize, wheat is one of the most important crops in the world (Shewry et al., 2002), widely used in human and domestic animal nutrition. Moreover, as the world population increases wheat and other crops became commercially more important.

In Portugal, wheat crops area suffered a dramatic decrease. Particularly, *Triticum durum* crop area was reduced 69% in 2011 due to adverse climatic conditions (INE, 2011). In fact, the decrease of *T. durum* production in Portugal in the last 10 years reached 75% (INE, 2011). Thus, it is mandatory to study this plant species through the characterization of *T. durum* distinct varieties available for crop production.

Inter Retrotransposons Amplified Polymorphism (IRAP) and Inter Simple Sequence Repeat (ISSR) PCR based techniques were developed by Kalendar et al. in 1999 and have been successfully used to characterize and distinguish cultivars/varieties from distinct plant species (reviewed in Bento et al 2011).

Wheat nutritional value comes from its dough used for making bread, pasta and other backed products (Shewry et al., 2002). This dough is washed, losing starch granules and water soluble constituents and the remaining rubbery mass is called Gluten. This mass contains between 75-85% proteins and 5-10% lipids (Wieser, 2007), constituting a good nutritional source.

Distinct food types are obtained from wheat dough based on gluten viscoelastic properties, which are mainly affected by the quality and strength of gluten proteins (Dong et al., 2010; Wieser, 2007). The prolamins are a group of seed storage proteins that play an important role in determining gluten viscoelastic properties. This class of proteins can be divided into two main groups: gliadins and glutenins. Gliadins are monomeric proteins soluble in aqueous solutions that contribute mainly to dough extensibility. On the other hand, glutenins are polymeric proteins soluble in alcohol solutions that play a major role in dough elasticity (Dong et al., 2010). Glutenins can be further divided into two other groups, according to their molecular weight: the high molecular weight subunits (HMW-GS) and the low molecular weight subunits (LMW-GS) (Dong et al., 2010; Zhang et al., 2011). The LMW-GS have the ability to form inter-molecular disulphide bonds with HMW-GS or other LMW-GS molecules, forming glutenin polymers responsible for gluten structure and properties (D'Ovidio and Masci, 2004).

2. Objectives

In this work we aim to characterize genomic variability among six *T. durum* commercial varieties used in Portugal through DNA fingerprinting PCR-based techniques such as IRAP (Inter-Retrotransponson Amplified Polymorphism) and ISSR (Inter-Simple Sequence Repeat).

Additionally, we analyzed molecular markers associated with gluten quality, focusing on the low molecular weight proteins due to the existence specific subunits directly related with gluten quality (D'Ovidio, 1993).

3. Materials and methods

3.1 Plant material and DNA isolation

The certified commercial varieties (Catálogo Nacional de Variedades 2012) of *Triticum turgidum* subs *durum* (AABB, 2n=4x=28), 'Celta', 'Hélvio', 'Marialva', and three other commercial varieties, 'Don Duro', 'Saragolla' and 'Severo' were used. Seeds were germinated and grown in controlled conditions with 16h light/8h dark (20ºC) cycles during a month, being then moved to a field greenhouse. Genomic DNA was isolated from fresh leaves of four individuals with six weeks old plants from each variety using Citogene DNA Purification Kit (Citomed).

3.2 Polymerase Chain Reaction (PCR)

IRAP (Inter Retrotransposons Amplified Polymorphism) was performed with primers designed for the LTR (Long Terminal Repeat) region of retrotransposons Angela and Fatima, while ISSR (Inter Simple Sequence Repeat) was performed with the anchored primer (AAG)6C (Table 1). IRAP and ISSR PCR reaction program consisted of: 2 min at 94ºC, followed by 30 cycles of 30s at 94ºC, 1 min at 50ºC with ramp +0.5ºC/s up to 72ºC; and terminal extension of 10min at 72ºC.

For the Low-Molecular-Weight Glutenin families (LMW-GS) two sets of primers were used: one to distinguish between the presence of LMW subunit 1 or LMW subunit 2 (associated with superior quality) (D'Ovidio, 1993), and another pair design to the conserved regions of LMW-GS genes to analyze the differences in total LMW-GS composition among the different varieties (Zhang et al., 2011) (Table 1). For LMW-GS amplifications PCR program consisted of: 3min at 95ºC, followed by 34 cycles of 45s at 95ºC, 1min at 60ºC, 45s at 72ºC, and with a terminal extension of 10min at 72ºC.

All PCR reaction consisted of a 40µl mix with: 23,2µl H2O, 4µl buffer 10xPCR, 1,2µl MgCl2 (50mM), 0,4µl dNTP's (0,25mM), 0,4µl Taq Polymerase (5U/µl), 0,4µl of each primer (forward and reverse) (100pmol/µl) and 10µl DNA (10ng/µl). PCR products were separated through horizontal electrophoresis (30µl of each reaction) in 1,7% agarose gel and stained with ethidium bromide. The gels were photographed using BioRad GEL DOC 2000.

Table 1 - Primers sequences used.

Retrotransposons			
	Angela	Fow	TAT GTT GTG TCA ACG CTT CC
	Fatima	Fow	TAG TCC GGC TAC TCA AGC AC
Microsatellite			
	(AAG)6C	Fow	AAG AAG AAG AAG AAG AAG C
LMW-Gs			
	D'Ovidio, 1993	Fow	CGT TGC GGC GAC AAG TGC AA
		Rev	GTA GGC ACC AAC TCC GGT GC
	Zhang et al., 2011	Fow	ATG AAG ACC TTC CTC GTC TTT G
		Rev	CAA CAT TGT CGC TGC ATC ACA T

4. Results

In this work different types of sequences were used to analyze the variability of distinct commercial varieties of durum wheat, namely repetitive sequences (retrotransposons and microsatellites) and coding sequences (low molecular weight glutenin *loci*).

4.1 Repetitive sequences analysis

IRAP - Retrotransposon Angela

The results obtained with the primer designed to the LTR of Angela retrotransposon are very informative and reveal the presence of polymorphisms even between individuals of the same commercial variety. This analysis clearly showed the existence of genomic variability within all analyzed varieties except in Hélvio and Severo varieties that presented banding profiles homogeny (Table 2).

Table 2: Number of bands present in the profiles obtained with Angela IRAP in all individuals from distinct varieties of *Triticum durum* analyzed.

Varieties / individuals	Celta	Hélvio	Marialva	Don Duro	Saragolla	Severo
No. of bands observed	7	6	8	12	12	12
Total no. of individuals analyzed	4	4	4	4	4	4
No, of polymorphic individuals	1	0	1	1	2	0
No. of bands in polymorphic individuals	7	-	7	12	12/13	-

In Celta variety there are three individuals with the same banding profile and one polymorphic individual (no.1) that revealed a unique pattern with three unique bands (~1100bp, ~1600bp and ~1650bp) (Figure 1, square and triangle). Within Marialva variety, three individuals presented the same banding profile, but individual no.1 revealed to be polymorphic with a profile missing one band with ~1100bp (Figure 1, square). Don Duro individuals banding profiles revealed that there are three individuals showing the same pattern and one polymorphic individual (no. 4) with two missing bands (~825bp and ~1700bp). Regarding Saragolla individuals it is possible to see that no.3 and no.4 show the same banding profile and individuals no.1 and no.2 preset one extra band with ~1650bp (Figure 1, circle), additionally in individual no.1 one band is missing ~950bp (Figure 1, star).

When comparing between different varieties it is possible to see that the total number of bands varies substantially, from a minimum of six bands obtained in all individuals of Hélvio variety to a maximum of 12 bands per individual profile in Don Duro, Saragolla and Severo (Table 2). Banding profiles presented in Figure 1 show that only three bands are common to all analyzed varieties (~700bp, ~1000bp, ~1400bp). Varieties Celta, Hélvio and Marialva show a smaller number of total bands (7, 6 and 8 respectively) and Don Duro, Saragolla and Severo show a much higher number of bands per profile (all with 12 bands). Those last three varieties reveal unique profiles showing differences in bands from 1500 to 1700bp (Figure 1, circle). Interestingly Marialva's individual no,1 shows the same banding pattern as the three non-polymorphic individuals of Celta, and Don Duro's no.4 show the same banding profile as Saragolla's no.1.

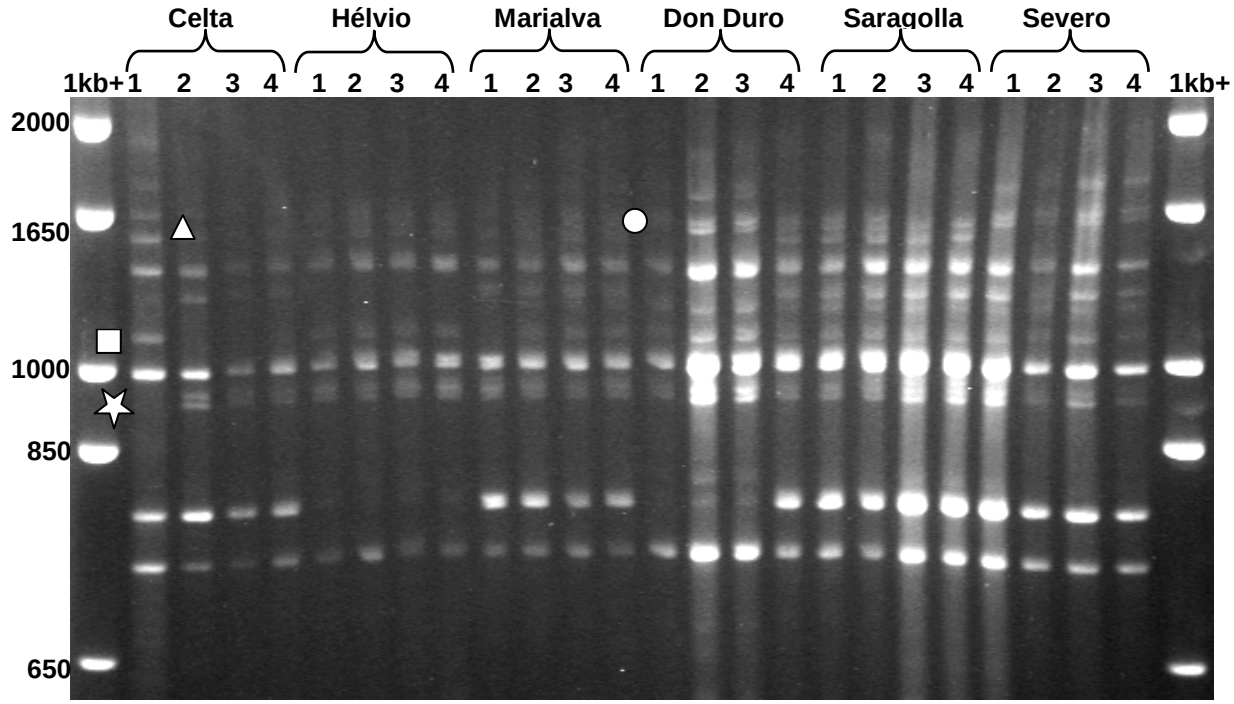

Figure 1: IRAP products obtained with primers to the LTR of retrotransposon Angela in *Triticum durum* cvs. Celta, Hélvio, Marialva, Don Duro, Saragolla and Severo (Numbers 1 to 4 represents different individuals of the same variety). Molecular marker 1kb+.

IRAP - Retrotransposon Fatima

The banding patterns obtained with the primer designed to the LTR of Fatima retrotranspopson are very similar between all individuals of the same variety and between all varieties analyzed (Figure 2). The Fatima IRAP common banding pattern is composed by 8 bands with the relative approximate sizes of 375bp, 400bp, 450bp, 475bp, 700bp, 750bp, 800bp and 1300bp. However, the band with approximately 700bp appears to be absent in the Don Duro, Saragolla and Severo varieties (Figure 2, triangle). Additionally, the band with ~550bp is only present in Hélvio variety and in three individuals of Don Duro variety (Figure 2, circle).

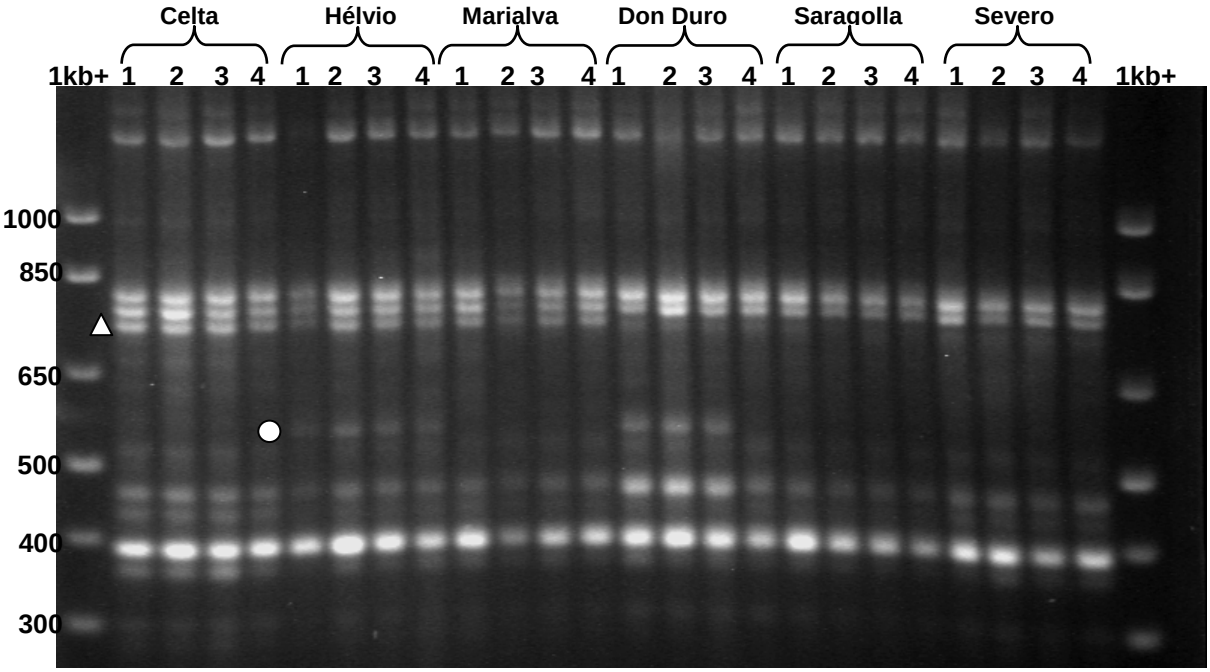

Figure 2: IRAP products obtained with primers to the LTR of retrotransposon Fatima in *Triticum durum* cvs. Celta, Hélvio, Marialva, Don Duro, Saragolla and Severo (Numbers 1 to 4 represents different individuals of the same variety). Molecular marker 1kb+.

ISSR - (AAG)6C

The banding patterns obtained with the anchored microsatellite primer (AAG)6C are presented in Figure 3 being very similar in all individual within the same variety and between varieties analyzed. This pattern comprehends 8 bands with the following approximately size: 275bp, 500bp, 625bp, 650bp, 800bp, 825bp, 1600bp and 1750bp. However, an additional band with ~350bp (Figure 3, star) is present in all individuals of Celta and Hélvio varieties and in only one Marialva individual (no.1). Furthermore, two bands (~900bp, Figure 3 circle; and ~1300bp, Figure 3 triangle) are present only in some individuals, one Celta (no.2), two individuals of Hélvio (no.3 and no.4), one of Marialva (no.1), one individual of Don Duro (no.2) and two individuals of Severo variety (no.1 and no.3).

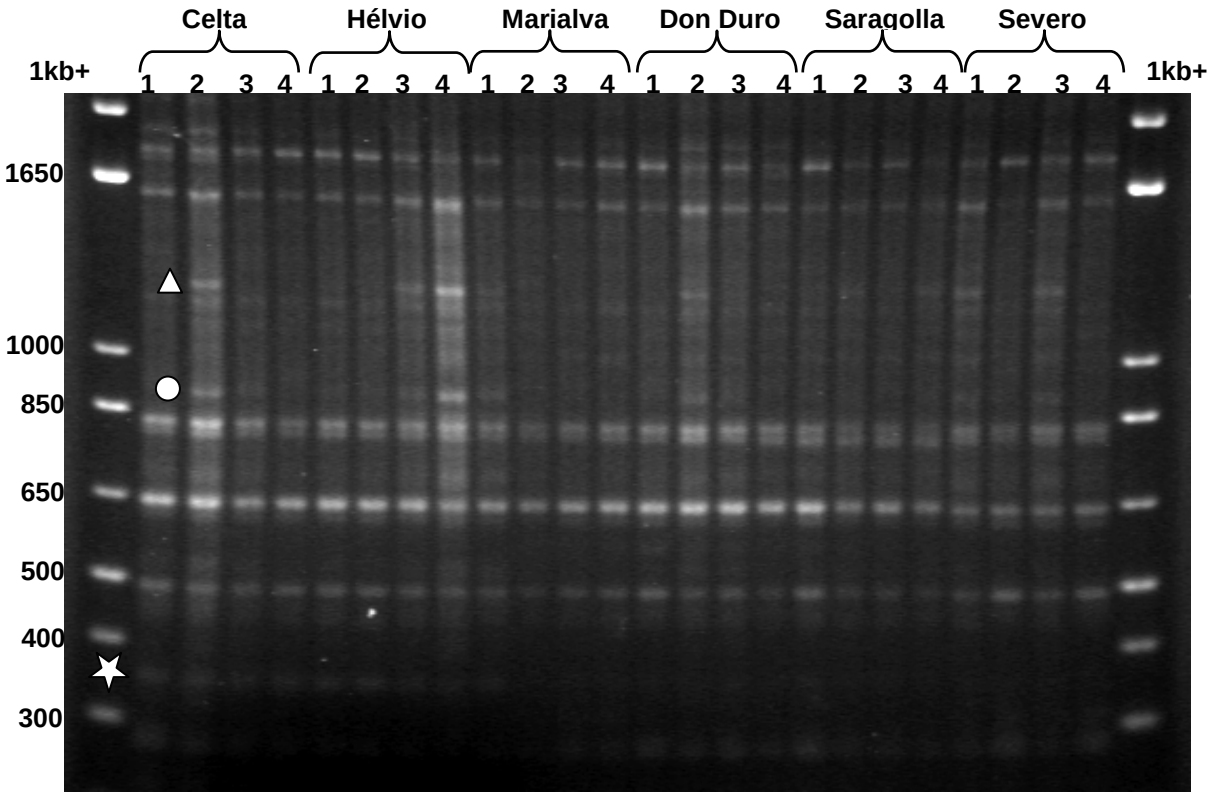

Figure 3: ISSR products obtained with primers to the anchored primer to microsatellite (AAG)6C in *Triticum durum* cvs. Celta, Hélvio, Marialva, Don Duro, Saragolla and Severo (Numbers 1 to 4 represents different individuals of the same variety). Molecular marker 1kb+.

4.2 Coding sequences analysis

Total LMW-GS composition

The results obtained with primers design to the conserved regions of LMW-GS genes show that all individuals from varieties Marialva, Saragolla and Severo, as well as three individuals of Celta variety and one of Don Duro variety share the same pattern composed by 6 bands with the relative sizes of 475bp, 525bp, 550bp, 650bp, 675bp and 800bp. The other individual of the Celta variety lack the referred 550bp band. The banding profile obtained in

Hélvio variety lacks the bands with 525bp and 800bp and shows a unique band with ~600bp (Figure 4, circle). All individuals from the variety Don Duro, except one (no.4) show only the band with approximately 500bp in the region between 475-550bp (lacking two bands in this region), sharing the rest of the common pattern (Figure 4, stars).

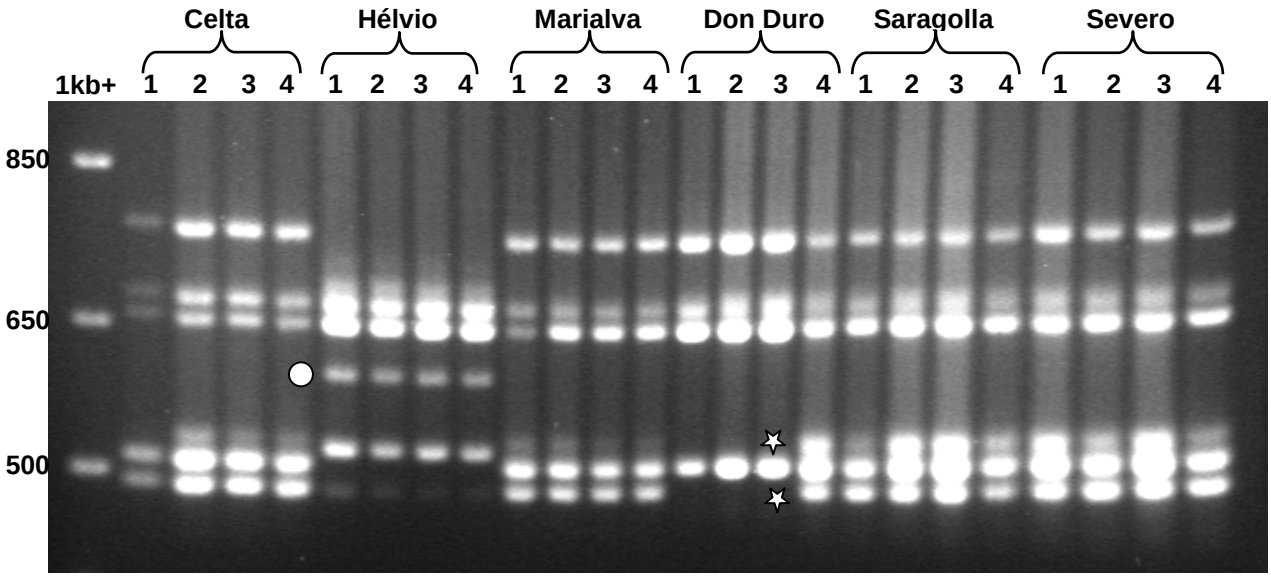

Figure 4: PCR products obtained with primers to analyze total LMW-GS composition in *Triticum durum* cvs. Celta, Hélvio, Marialva, Don Duro, Saragolla and Severo (Numbers 1 to 4 represents different individuals of the same variety). Molecular marker 1kb+

LMW-G subunit 1 or 2

The banding patterns obtained in distinct durum wheat varieties with primers to amplify LMW-G subunit 1 and 2 show two main bands. The lower fragment with approximately 1000bp is common to both subunits (LMW-G subunit 1 and 2). The larger fragment allows to distinguish between the presence of LMW-G subunit 1 (LMW-1) or 2 (LMW-2) since LMW-2 fragment is larger (~1200bp) than the LMW-1 (~1150bp) (D'Ovidio, 1993). All individuals of Hélvio variety and three individuals of Marialva, Don Duro, Saragolla and Severo show the larger fragment (~1200bp), representative of LMW-2. The other individual of Marialva variety (no.1) and all the Celta individuals show the smaller fragment (~1150bp) corresponding to LMW-1 (Figure 5).

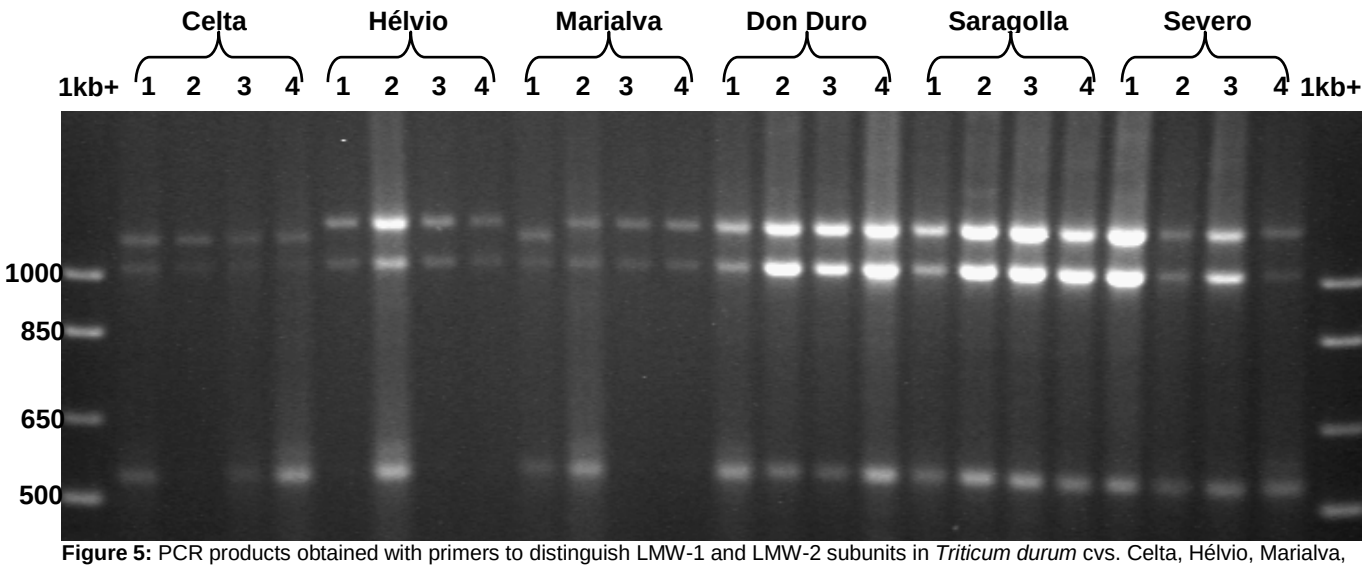

Figure 5: PCR products obtained with primers to distinguish LMW-1 and LMW-2 subunits in *Triticum durum* cvs. Celta, Hélvio, Marialva, Don Duro, Saragolla and Severo (Numbers 1 to 4 represents different individuals of the same variety). Molecular marker 1kb+.

5. Discussion and conclusion

5.1 Genomic variability of repetitive sequences

IRAP and ISSR results presented revealed differences between all *T. durum* varieties analyzed and even intra-variety variability. This variability observed between individuals of the same variety was not expected since they are reproduced through self-fertilization and represent distinct commercial varieties. While Fatima retrotransponson revealed no differences within the same variety and show only a few differences between distinct varieties, Angela retrotransposon revealed a considerable amount of differences between varieties and also within distinct individuals of the same variety. Microsatellite analysis using the (AAG)6C primer showed small differences both within and between varieties. Thus, IRAP and ISSR techniques are very useful to characterize and distinguish different commercial durum wheat varieties, with IRAP technique revealing more genomic variability than ISSR (Table 3).

Table 3 – Summary of variability in banding profiles identified by IRAP and ISSR techniques.

Sequences	**Retrotransposons**		**Microsatelites**
N. of bands	*Angela*	*Fatima*	*(AAG)6C*
Total	17 (100%)	9 (100%)	11 (100%)
Conserved	3 (17,65%)	7 (77,78%)	8 (72,72%)
Polymorphic	14 **(82,35%)**	2 **(22,22%)**	3 **(27,27%)**

The results presented also show that there are some differences among individuals from the same variety, but as expected those are less common than the ones identified between different varieties. These differences prove the existence of variability between the analyzed commercial varieties which can be promising for instance, in terms of seed quality, and tolerance to adverse environmental conditions.

The divergences among individuals can be related with the possibility of seeds being wrongly misidentification. For instance individual no.1 of Marialva variety shows the same banding profile as the three non-polymorphic individuals of Celta and the same is revealed using Angela retrotransposon and in the analysis of LMW subunit 1. Additionally, individual no.4 of Don Duro variety shows the same banding profile as Saragolla individuals for Angela and Fatima retrotransponsons and also in LMW-GS family pattern.

5.2 Low molecular weight glutenins variability

The analysis of patterns obtained with primers for LMW-GS protein family showed that Hélvio present the most distinctive pattern, being also detected differences between other varieties. Within the same variety differences were detected between Celta and Don Duro individuals.

As previously referred, the LMW subunit 2 is linked to a better dough quality (D'Ovidio, 1993) being expected that all individuals from all commercial varieties present this subunit. However, the analysis performed revealed that all individuals of Celta variety and one of Marialva (no.1) have LMW subunit 1.

5.3 Future perspectives

The results obtained will most certainly permit the development of primers to clear and readily distinguish between durum wheat varieties as well as to confirm their homogeneity through an easy analysis performed by single PCR reactions.

Regarding seed storage protein *loci* we hope to expand the study developed to HMW-GS and gliadins using primers for the subunits related to better dough quality, in order to better identify and characterize commercial varieties with superior quality traits.

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