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RESEARCH ARTICLE

Physical localization of NORs and ITS length variants in old Portuguese durum wheat cultivars

ANA CARVALHO, HENRIQUE GUEDES-PINTO and JOSÉ LIMA-BRITO*

Institute of Biotechnology and Bioengineering (IBB), Centre of Genomics and Biotechnology (CGB), University of Tras-os-Montes and Alto Douro, P. O. Box 1013, 5001-801 Vila Real, Portugal

Abstract

The variation at the internal transcribed spacer (ITS) region of the ribosomal DNA has been correlated with the number of nucleolar organizer regions (NORs) in some plant species. Besides, the number of NORs might influence the rate of homogenization of the rDNA repeats. In recent studies, ITS length variants were detected in bread wheat cultivars but no reports about their presence in durum wheat were found. In the present study, we localized and identified the NORs of 51 old Portuguese durum wheat cultivars by using sequential silver staining and fluorescence *in situ* hybridization performed with the pTa71 rDNA probe. We also detected ITS length variants by PCR-RFLP. No variation at the number of Ag-NORs per metaphase was found among the 51 durum wheat cultivars, but the PCR-RFLP technique carried out with the restriction enzyme *Hpa*II, allowed the detection of ITS length variants among them. The molecular data was used in order to establish the genetic relationships among cultivars and botanical varieties of durum wheat. The knowledge of this feature could be useful for future design of breeding strategies, involving this collection that constitutes an excellent repository of germplasm in Portugal.

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Introduction

One wheat repeat unit of the ribosomal rDNA (rDNA) is constituted by the coding regions (18S-5.8S-26S rRNA genes) and by the spacers: internal transcribed spacer (ITS), which includes the ITS1 and the ITS2 that flank the 5.8S rRNA gene, and the intergenic spacer (IGS) that relies between 26S and 18S rRNA genes (Barker *et al.* 1988). The rDNA spacers have been considered highly variable regions with rapid rates of evolution (Barker *et al.* 1988; Baldwin *et al.* 1995). In the recent years, ITS length variants have been identified for different plant species, including bread wheat (Baldwin *et al.* 1995; Álvarez and Wendel 2003; Nalini *et al.* 2007; Saini *et al.* 2008; Carvalho *et al.* 2009a). However, for durum wheat there were no reports about ITS length variants. The ITS polymorphism might occur at different taxa and could provide a useful technique for phylogenetic, evolutionary and biogeographical studies (Nwakanma *et al.* 2003). Previously, our group demonstrated the utility of ITS PCR-RFLP markers for the assessment of genetic diversity in old Portuguese bread wheat cultivars, and for the establishment of genetic relationships among those botanical varieties (Carvalho *et al.* 2009a). The durum wheat germplasm analysed here, also belongs to the above Portuguese wheat collection, which constitute an excellent repository of genetic variability in our country.

With the present study, we intend to determine the physical localization and identification of NORs in 51 old Portuguese durum wheat cultivars using sequential silver nitrate staining and fluorescence *in situ* hybridization (FISH) performed with the probe 45S rDNA – pTa71 (Gerlach and Bedbrook 1979), and to screen for the presence of ITS length variants by using the PCR-RFLP methodology.

Materials and methods

Plant material

The old Portuguese durum wheat cultivars were kindly provided by the National Plant Breeding Station (ENMP, Elvas, Portugal) (table 1). Homonym cultivars were denoted as (a),

*For correspondence. E-mail: jbrito@utad.pt.

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Table 1. Passport data of the old Portuguese durum wheat cultivars ($2n = 4x = 28$; AABB).

Cultivar passport code	Botanical variety	Cultivar name
T. turgidum subsp. *dicoccum*		
2	*aestivum*	Alentejo
9	*false-jodurum*	Asa de Corvo (a)
10	*jodurum*	Asa de Corvo (b)
11	*pseudomirabile*	Bagudo
63	*pseudosalomonis*	Pombinho
76	*mertensii*	Rubião de Barba Preta
86	*megalopolitanum*	Sicílio
T. turgidum subsp. *durum*		
3	*africanum*	Alexandre
6	*africanum*	Anafil Escuro
21	*africanum*	Durázio Molar
35	*africanum*	Gigante Inglês
68	*africanum*	Pragana Preta
8	*reichenbachii*	Argelino
22	*reichenbachii*	Durázio Molar Glabro
45	*reichenbachii*	Marquês
65	*reichenbachii*	Preto Amarelo
67	*reichenbachii*	Preto Algarvio (b)
12	*coerulescens*	Barba de Lobo (a)
13	*coerulescens*	Barba de Lobo (b)
15	*leucurum*	Branco
17	*leucurum*	Candeal
16	*affine*	Candeal de grão escuro
41	*affine*	Lobeiro
78	*affine*	Russo
90	*affine*	Tremês Rijo
18	*niloticum*	Corado
19	*pseudosalomonis*	Dezassete
20	*melanopus*	Durazia Rijo
25	*provinciale*	Entrelargo do Montijo (a)
27	*provinciale*	Entrelargo do Montijo ©
26	*obscurum*	Entrelargo do Montijo (b)
61	*obscurum*	Mourisco preto de grão escuro
28	*libycum*	Escuro
39	*libycum*	Javardo
62	*libycum*	Mourisco Preto
42	*murciense*	Lobeiro Ruivo
57	*murciense*	Mourisco Ruivo (a)
58	*murciense*	Mourisco Ruivo (b)
98	*murciense*	Vermelejoilo
99	*murciense*	Vermelho Fino
54	*fere-alexandrinum*	Mongia (a)
55	*fere-alexandrinum*	Mongia (b)
56	*fere-alexandrinum*	Mongia de grão escuro
60	*fere-alexandrinum*	Mourisco Fino
89	*alexandrinum*	Tremês Preto
59	*erythromelan*	Mourisco
66	*alboprovinciale*	Preto Algarvio (a)
72	*apulicum*	Raspinegro
85	*hordeiforme*	Santa Marta
97	*triste-leucomelan*	Verdial Rijo
102	*durum*	Caxudo

Preparation of chromosome spreads, silver nitrate staining and FISH.

(b) and/or (c). According to the passport data, 51 cultivars belong to 27 different botanical varieties (table 1). Regarding their geographical origin/distribution areas, these cultivars might be considered sympatric across the centre and south of Portugal.

The germination of seeds, collection of root-tips and preparation of chromosome spreads; the sequential technique of silver nitrate staining and FISH, as well as the detection of the cytogenetic results, were performed as described in Carvalho *et al.* (2010).

ITS rDNA amplification and PCR-RFLP

Genomic DNA was extracted from young leaves by a CTAB based protocol (Doyle and Doyle 1987). The rDNA ITS1-5.8S-ITS2 region was amplified as a single molecule using the primers ITS-4 (White *et al.* 1990) and a modified primer (GTCCACTGAACCTTATCATTTAG) reported by Urbatsch *et al.* (2000). The amplification reactions (final volume of 50 μL) and conditions were those as described in Carvalho *et al.* (2009a). Ten μL of each PCR product were analysed after electrophoresis on a 1.5% agarose gel and staining with ethidium bromide. The remaining PCR product (40 μL) was digested with 10 units of each restriction enzyme (*Alu*I, *Hpa*II, *Rsa*I and *Taq*I) in a 30 μL PCR-RFLP reaction, following manufacturer's instructions. The PCR-RFLP products were separated after electrophoresis on 2% agarose gels at a constant voltage of 90 V. Bands were visualized after staining with ethidium bromide and photographed with a CCD video imager (Vilber Lourmat, Eberhardzell, Germany). The size of the restricted products was estimated with the molecular weight marker GeneRuler 100-bp DNA Ladder Plus (Fermentas, Burlington, USA). Only the clearly amplified fragments were scored.

Statistical analysis

Despite their co-dominant nature, the ITS PCR-RFLP markers were analysed by the presence/absence of bands, in order to be used for the elaboration of a binary matrix and the subsequent construction of a genetic similarity dendrogram using the unweighted pair group method with arithmetical averages (UPGMA), the Jaccard coefficient (Jaccard 1908) and the module of sequential agglomerative hierarchical nested (SAHN) with the NTSYSpc version 2.02 software (Rohlf 1988). A cladogram and a phylogram based on Nei's genetic distance (Nei 1987) and in the neighbour-joining method (Saitou and Nei 1987) were also constructed with the software POPGENE 1.32 (Yeh *et al.* 1999) and TreeView (Page 1996) with the purpose of evaluating the genetic relationships among 27 botanical varieties of durum wheat.

Results

Physical localization and identification of NORs

The sequential technique of silver nitrate staining and FISH was successful in identification of four Ag-NORs per metaphase cell in all durum wheat cultivars (figure 1a). The Ag-NORs were located on the short arms of the chromosome pairs 1B and 6B (figure 1, a & c). The satellite chromosomes were identified after comparison with NOR patterns

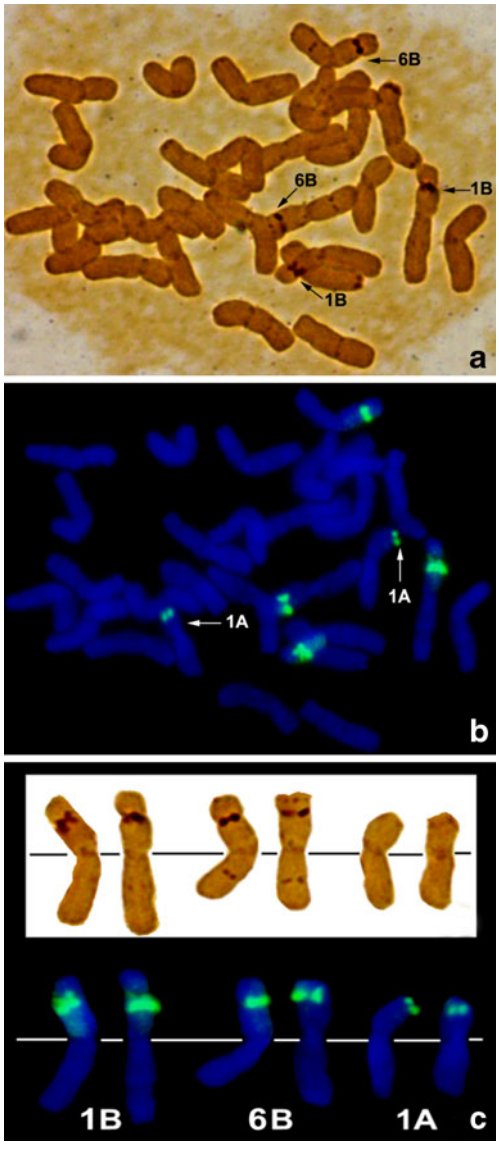

Figure 1. Metaphase cell of the durum wheat cultivar 'Barba de Lobo b' after a) silver nitrate staining, showing four Ag-NORs (arrows); b) FISH performed with the 45S rDNA probe, pTa71 (green), which confirmed the NORs location and detected two additional rDNA loci (arrows); c) identification of the NORs and/or rDNA loci.

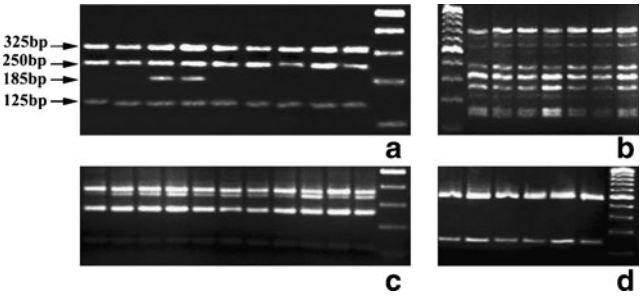

Figure 2. ITS PCR-RFLP patterns produced by: a) *Hpa*II; b) *Taq*I; c) *Alu*I, and d) *Rsa*I, among the 51 durum wheat cultivars. Arrows indicate both the monomorphic and the polymorphic bands of the two *Hpa*II patterns. The first (b) or the last (a, c and d) lane of each gel correspond to the molecular weight marker GeneRuler 100-bp DNA Ladder Plus (Fermentas, Burlington, USA).

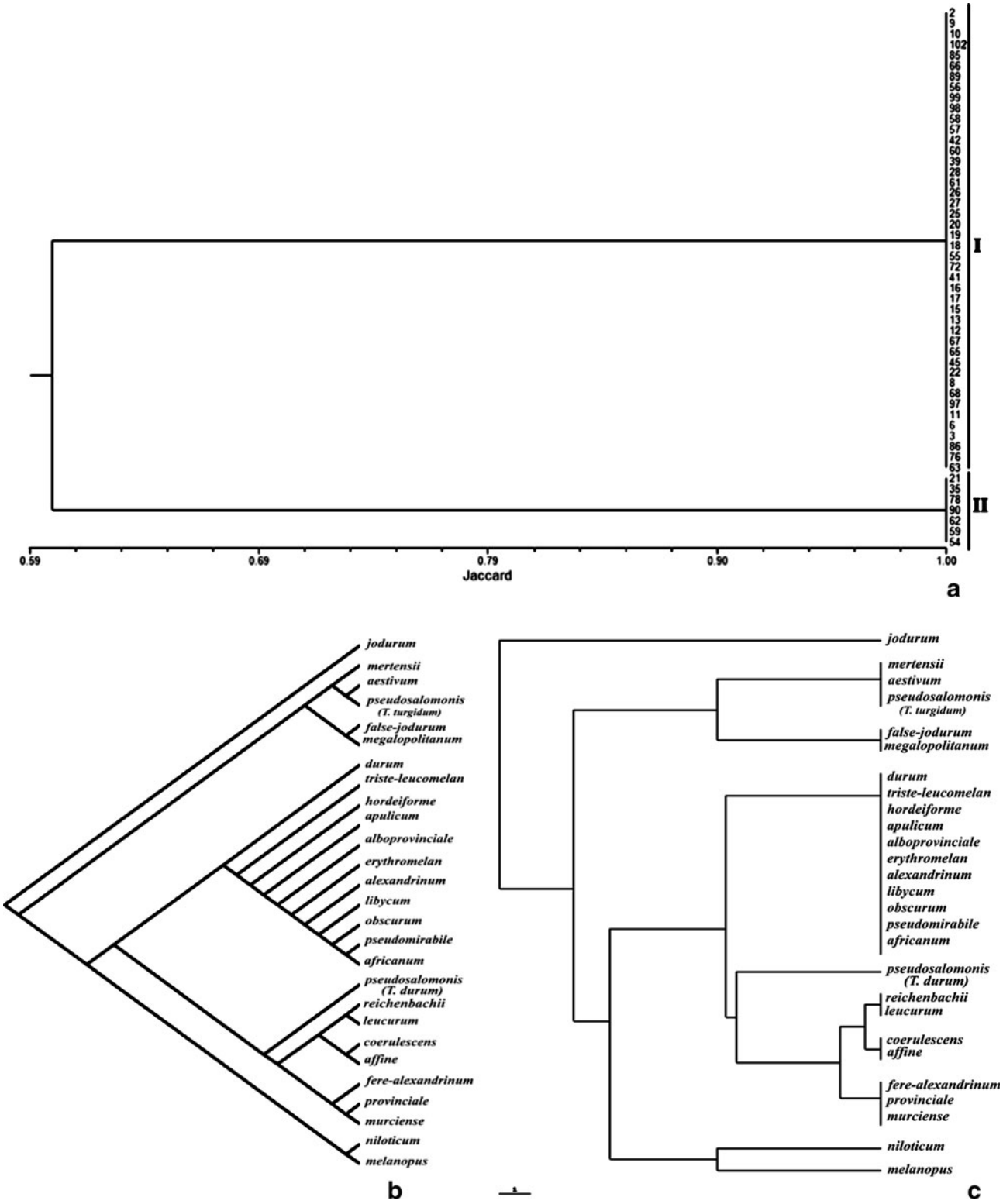

Figure 3. (a) UPGMA dendrogram of genetic similarity among 51 durum wheat cultivars, (b) cladogram, and (c) phylogram of the Portuguese durum wheat botanical varieties based on the ITS PCR-RFLP data produced by *Hpa*II.

previously described by Amado *et al.* (1997) and Silva *et al.* (2008). Following FISH, we detected two additional rDNA loci on the chromosome pair 1A that were transcriptionally inactive (negative staining with silver nitrate; figure 1b).

ITS variants detected by PCR-RFLP

The 51 durum wheat cultivars showed a 700-bp PCR product of invariant length. Except for the *Hpa*II (figure 2a), the remaining enzymes failed on the detection of ITS length variants (figure 2, b, c and d).

The *Hpa*II enzyme produced two ITS PCR-RFLP patterns among the 51 durum wheat cultivars: the most frequent (present in 86.28% of the cultivars) was composed by three monomorphic bands with 325 bp, 250 bp and 125 bp, and the lowest frequent (13.72%), was constituted by the three monomorphic bands and one additional polymorphic band of 185 bp (figure 2a). In total, the *Hpa*II enzyme detected two polymorphic fragments among five amplified ones, resulting in 40% of ITS polymorphism.

Genetic relationships estimated by ITS PCR-RFLP

To determine the genetic relationships among the 51 durum wheat cultivars based on ITS PCR-RFLP data achieved with the *Hpa*II, a binary matrix to construct a genetic similarity UPGMA dendrogram was elaborated (figure 3a). As reported previously, this enzyme detected 40% of ITS polymorphism, corroborating the range of genetic similarity among the 51 durum wheat cultivars (60%) that could be observed in figure 3a. The topology of the dendrogram was concordant with the two *Hpa*II patterns, once the durum wheat cultivars were clustered in two main groups; one composed of 44 cultivars and the other by seven cultivars that showed the polymorphic band of 185 bp (figures 2 and 3a).

To estimate the phylogenetic relationships among the 27 botanical varieties based on the ITS PCR-RFLP data, a cladogram (figure 3b) and a phylogram (figure 3c) were constructed. Both the cladogram and the phylogram clustered the botanical varieties in two main groups, except for *jodurum* (*T. turgidum* subsp. *dicoccum*) which constituted a branch (figure 3, b & c). A cladogram might provide the evolutionary paths of the taxonomic units, and it could be taken as an inferred phylogenetic tree, despite the unknowable temporal sequence of the events. The root of each cladogram could be considered the most recent common ancestor. Thus, based on the present data, we suggest that the botanical variety *jodurum* evolved directly from the common ancestor (figure 3, b & c). The remaining varieties from *T. turgidum* subsp. *dicoccum*, except for *pseudomirabile*, seem to diverge from an independent event which originated one of the main groups. At some extent, the ITS PCR-RFLP markers discriminated the botanical varieties of *T. turgidum* subsp. *dicoccum* from those of *T. turgidum* subsp. *durum*.

Discussion

Cytogenetic analysis

In all the old Portuguese durum wheat cultivars we detected four Ag-NORs per metaphase located on the short arm of the chromosome pairs 1B and 6B. These results agreed with those previously reported by other authors (Cermeño *et al.* 1984, 1987; Lacadena *et al.* 1984; Lima-Brito *et al.* 1998). Additionally, the NOR banding pattern achieved here was coincident with that described for the chromosomes 1B and 6B by Amado *et al.* (1997) and Silva *et al.* (2008), being helpful for the identification of the satellite chromosomes. We also detected two additional rDNA loci after FISH, corresponding to the chromosome pair 1A, that despite its ability for organizing nucleoli (Crosby 1957; Bhowal 1972), were transcriptionally inactive in all durum wheat cultivars studied here.

According to Flavell (1980), the number of NORs correspond to the number of multiple ITS sequences found in the same taxon. Our results were consistent with this assumption, since the patterns detected here (figure 2a) showed a maximum of four fragments, corresponding to the number of Ag-NORs detected per metaphase in all durum wheat cultivars (figure 1a). The localization of the NORs in the chromosome could also affect the rate of homogenization of the rDNA repeats (Saghai-Maroof *et al.* 1984).

ITS length variants

As far as we know, this is the first report about the detection of ITS length variants in durum wheat. ITS length variants were previously detected in bread wheat using PCR-RFLP (Nalini *et al.* 2007; Carvalho *et al.* 2009a). In our previous study performed in 48 old Portuguese bread wheat cultivars, we found out nine ITS PCR-RFLP patterns with the enzymes *Alu*I, *Hpa*II and *Taq*I (three patterns per enzyme), and 50% of ITS polymorphism (Carvalho *et al.* 2009a). In the present study, 51 durum wheat cultivars showed 40% of ITS variation, and this lower level of polymorphism could be due to the absence of the D genome. Additionally, the rDNA multigenic family is highly prompt to concerted evolution, leading to the homogenization of the sequences and number of repeats, including those of the spacer regions, within an individual (Wendel 2000; Saini *et al.* 2008), decreasing the ITS variation. Low ITS variation was previously reported for Triticeae (Zhang *et al.* 2002) and other taxa such as Cucurbitaceae (Jobst *et al.* 1998); Oleaceae (Jeandroz *et al.* 1997), and *Vigna* (Saini *et al.* 2008). Besides, plant species with a reduced number of NORs, such as durum wheat, could have a faster rate of homogenization of the rDNA repeats contributing for the low ITS variation.

Nalini *et al.* (2007) found out that the polymorphic ITS fragments were correlated with quantitative trait loci (QTL) such as the spike size and the number of spikelets per spike. Thus, the polymorphic ITS fragments detected here should

be further cloned and sequenced in order to screen for interesting agronomic traits with potential use in wheat breeding.

Genetic relationships estimated by ITS PCR-RFLP

The ITS sequences have been considered excellent markers for the establishment of phylogenies in Triticeae and other plant species (Hsiao *et al.* 1994, 1995; Baldwin *et al.* 1995; Goel *et al.* 2002; Sharma *et al.* 2002; Zhang *et al.* 2002; Álvarez and Wendel 2003).

ITS PCR-RFLP markers revealed a lower percentage of polymorphism when compared with other DNA markers such as ISSRs (see Carvalho *et al.* 2009b). However, they constitute feasible and specific tools for the assessment of genetic variation among the durum wheat cultivars and a higher taxa, such as botanical variety, being useful for the estimation of phylogenies.

The knowledge of the genetic relationships and phylogenies among the durum wheat cultivars and their botanical varieties might contribute for the designing of intraspecific crosses between the genotypes studied here, with potential interest for wheat improvement.

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