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Communication

Molecular Polymorphisms in Tunisian Pomegranate (*Punica granatum* L.) as Revealed by RAPD Fingerprints

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Dedicated to the Memory of Professor Mohamed Marrakchi, who passed away in April 2008

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Abstract: The genetic diversity among Tunisian pomegranate cultivars has been investigated. Using universal primers, the random amplified polymorphic DNA (RAPD) method was used to generate banding profiles from a set of twelve cultivars. Data was then computed with appropriate programs to construct a dendrogram illustrating the relationships between the studied cultivars. Our data proved the efficiency of the designed method to examine the DNA polymorphism in this crop since the tested primers are characterized by a collective resolving power of 12.83. In addition, the cluster analysis has exhibited a parsimonious tree branching independent from the geographic origin of the cultivars. In spite of the relatively low number of primers and cultivars, RAPD constitutes an appropriate procedure to assess the genetic diversity and to survey the phylogenetic relationships in this crop.

Keywords: pomegranate; *Punica granatum* L.; diversity; RAPD

1. Introduction

The pomegranate (*Punica granatum* L.), a woody perennial shrub or small tree, is native of Persia [1] and is one of the oldest known edible fruit trees. It has been cultivated extensively in the Mediterranean countries such as Tunisia, Turkey, Egypt, Spain, and in some extent also in California, China, Japan and Russia [2]. The pomegranate is cultivated for its edible fruits and/or for decorative purposes. Its utilization consists of a large number of horticultural varieties mainly characterized by fruits' traits (rind color, seed color, taste, shape). In Tunisia, the pomegranate has been traditionally cultivated since ancient times under diverse climatic conditions [1]. More than 60 local varieties have been listed by Mars [3]. Their denominations reflect mainly origin area, the shape or the fruit color [4,5]. To date, characterization of Tunisian pomegranate has been accomplished by the use of morphological descriptors [3]. Thus, Mars has reported a list of 29 traits useful for scoring of *Punica granatum* L. cultivars [3]. Although classical phenotypic features are still extremely useful, the genotypic identification efficiency may be reduced by several effects such as age, development stage and environmental factors [6]. Moreover, cytoplasmic genetic effects on morphology constitute an additional factor contributing to ambiguities encountered in the results of key-derived genotype classification. This leads to a lack of information and a decrease in the success of clone differentiation based on morphological characteristics [7]. To overcome these aspects, a large panel of PCR based methods, such as AFLP, RAPD, ISSR and SSR, have been developed, with wide range of complexity, to examine the genetic diversity between and within fruit species. Diversity in *Punica granatum* has been investigated by RAPD [8-10] and AFLP [11,12], and recently 18S-28S rDNA intergenic spacer-RFLP has been used for pomegranate cultivar identification [13]. RAPD was largely used for fingerprinting accessions and to estimate genetic relatedness in germplasm collections [7,14-19], given its simplicity, efficiency and especially the non-requirement of DNA sequences. In the present work, we report the usefulness of the RAPD method to assess the molecular genetic diversity of Tunisian pomegranate.

2. Results and Discussion

2.1. Primers and Resolving power (Rp)

Among the 12 primers tested, only four generated unambiguously reproducible bands after two PCR runs (Table 1). These are: OPA04, OPA19, OPH08 and OPH09. The remaining ones all yielded weak and/or non-reproducible bands. Taking into account the four retained primers, a total of 29 bands have been generated with a mean of 7.25 per primer (Table 2). The number of generated bands varied from 3 to 11, with 0.5 to 3 kb size range. OPH07 and OPA19 primers generated nine and eight polymorphic bands, respectively, while amplification with OPH08 and OPA04 gave only four and three polymorphic bands, respectively. The total number of polymorphic bands produced using four RAPD primers was 24 out of 29. This led to relatively higher Rp values of the former primers when compared to the latter ones. Thus, we may assume that the designed procedure is a powerful tool to examine the genetic diversity in pomegranate germplasm.

Table 1. Primers' sequences, number of polymorphic bands and resolving power.

Primer	Sequence (5'−3')	Amplified bands		
		Total	Polymorphic	Rp
OPA 04	AATCGGGCTG	3	3	1.333
OPA 19	CAAACGTCGG	10	8	4.832
OPH 07	CAAACGTCGG	11	9	4.666
OPH 08	GAAACACCCC	5	4	2.000
	Total	29	24	12.831

2.2. Relationships between Cultivars

The generated RAPD profiles were used to assess similarities among the studied cultivars by genetic distances (Table 2) and dendrogram (Figure 1). Genetic distance ranged from 0.042 to 0.792, with a mean of 0.384. The lowest genetic distance (0.042) is registered between *'Garoussi 2'* [GR2] and *'Bellahi 1'* [BL1], suggesting their close relatedness, whereas *'Mezzi 1'* [MZ1] and *'Gabsi 9'* [GB9] cultivars seem to be the most divergent, since they have exhibited the highest genetic distance (0.792). All the remaining cultivars shared intermediate genetic distance values.

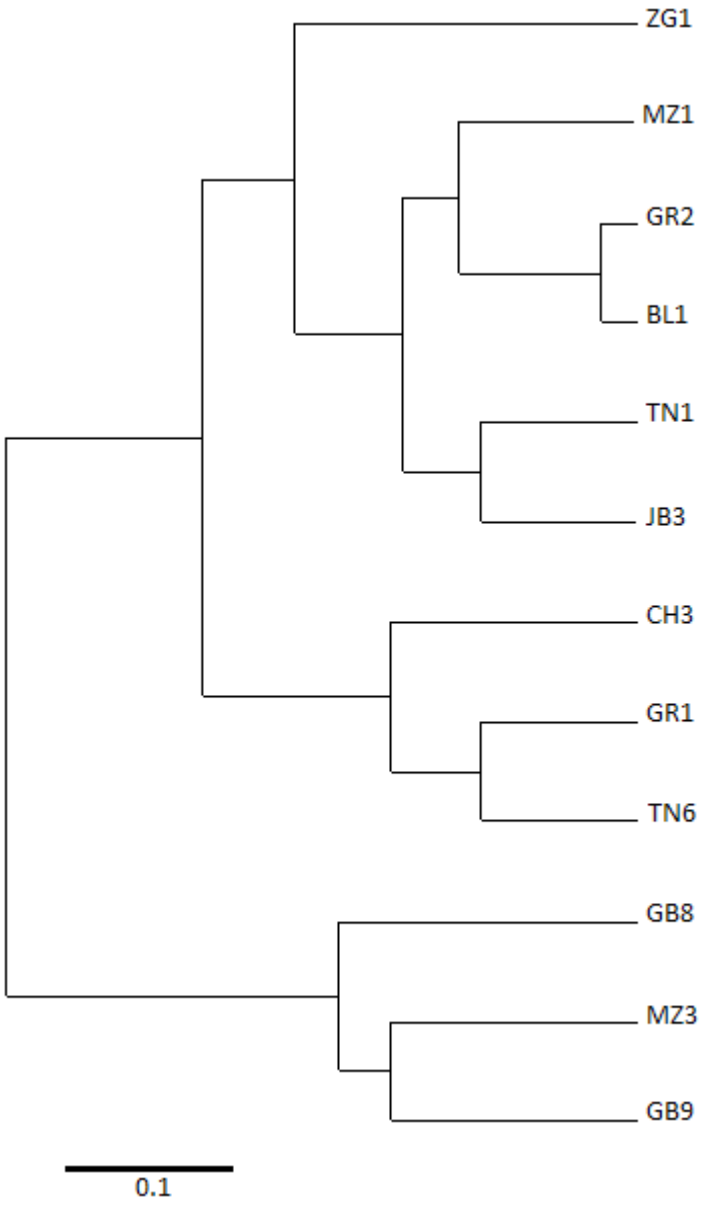
Table 2. Genetic distances calculated on their RAPD fingerprints.

GR2	0											
GR1	0.292	0										
BL1	0.042	0.333	0									
ZG1	0.292	**0.500**	0.333	0								
MZ3	0.417	0.292	0.458	0.375	0							
TN6	0.292	0.167	0.250	0.417	0.208	0						
TN1	0.208	0.333	0.167	0.333	0.458	0.250	0					
JB3	0.208	0.250	0.250	0.250	0.292	0.167	0.167	0				
MZ1	0.208	**0.500**	0.167	0.417	**0.625**	0.417	0.250	0.333	0			
GB9	**0.667**	0.458	**0.708**	0.375	0.250	0.458	**0.542**	0.458	**0.792**	0		
GB8	**0.625**	**0.583**	**0.667**	0.333	0.292	**0.500**	**0.583**	0.417	**0.667**	0.292	0	
CH3	0.458	0.250	0.417	**0.667**	0.375	0.250	0.333	0.417	**0.583**	0.375	**0.667**	0
Cultivars	GR2	GR1	BL1	ZG1	MZ3	TN6	TN1	JB3	MZ1	GB9	GB8	CH3

Genetics distances are supported by the resulting dendrogram shown in Figure 1. Two main groups could be identified. The first one is made up of three cultivars: *'Gabsi 9'* and *'Mezzi 3'*, related to *'Gabsi 8'*. The remaining cultivars were divided into two sub-groups. Cultivars belonging to the second main group are grouped in two sub-clusters. The first is made up of three cultivars: *'Garoussi 1'*, *'Tounsi 6'* and *'Chelfi 3'*. Inside the second one, *'Zaghouani 1'* cultivar was distantly related to the five remaining sub-clustered cultivars.

Opportunely our data portrayed the advantages of the RAPD method in the exploration of pomegranate polymorphisms at the DNA level. This is strongly supported by recent works dealing with the usefulness of RAPD to investigate pomegranate diversity [8-10].

Figure 1. UPGMA dendrogram of 12 Tunisian pomegranate cultivars showing phenetic relationships of estimated from RAPD data.



The tested universal primers are characterised by a collective Rp of 12.83. The Rp values, as well as the number of generated polymorphic markers, were relatively lower than revealed in other fruit species such as figs [20]. The following hypotheses could be forwarded to explain this result: (i) the small number of tested primers together with the number of cultivars analysed. as larger diversity could be detected by using much more primers and cultivars [8]; (ii) the farmers' high selective pressure applied in this crop and its vegetative propagation. This second hypothesis is strongly supported since exchanges of cuttings made by farmers occurred over many decades would facilitate

the assignment of locally adapted and presumably genetically identical ecotypes. The little divergence within pomegranate cultivars suggests the presence of a common genetic basis in the Tunisian pomegranate germplasm despite morphological dissimilarities among ecotypes [3]. This assumption is strongly supported since the cultivar clustering (Figure 1), which is made independently from their geographic origin. This is well exemplified in the case of *Garoussi 2* and *Bellahi 1*, the most closed cultivars, together with the *Mezzi 1* [MZ1] that are originated from the Chott-Mariem and Tozeur groves, respectively.

3. Experimental Section

3.1. Plant Material

Twelve Tunisian pomegranate cultivars (Table 3) were collected throughout the major pomegranate growing regions and are maintained at the national collection of pomegranate located at Gabès, in southern of Tunisia [4,5]. Young leaves randomly sampled from adult trees of each cultivar, were washed in water and frozen at −80 °C untill DNA extraction.

Table 3. List of 12 Tunisian pomegranate cultivars, their origin and main characteristics.

Cultivar	Label	Origin	Main characteristics		
			Taste	Rind color	Arils color
Gabsi 8	GB8	Kettana	Sweet	Red	Redish pink
Gabsi 9	GB9	Zerkine II	Sweet	Red	Redish pink
Tounsi 1	TN1	Testour	Sweet	Red	Dark red
Tounsi 6	TN6	Tozeur	Sweet	Pale red	Red
Chelfi 3	CH3	Testour	Sweet	Red	Redish pink
Mezzi 1	MZ1	Tozeur	Sour	Pale red	Dark red
Mezzi 3	MZ3	Tozeur	Sour	Pale red	Redish pink
Jebali 3	JB3	El Alia	Sweet	Dark red	Redish pink
Garoussi 1	GR1	Mareth	Sweet	Red	White pink
Garoussi 2	GR2	Chott Mariam	Sour	Pale red	Red
Zaghouani 1	ZG1	Zaghouan	Sweet	Dark red	Redish pink
Bellahi 1	BL1	Tozeur	Sweet	Red green	White pink

3.2. DNA Extraction

Total DNA was extracted according to the procedure of Dellaporta *et al*. [21]. The quality and quantity of DNA were checked by analytic electrophoresis in a 1% agarose gel containing ethidium bromide (0.5 mg ml^{-1}) in 1×TBE as described by Sambrook *et al*. [22].

3.3. Primers and PCR Assays

After first step of primer screening including 12 universal primers from kits A and H (Operon Technologies, Alameda, CA, USA) and four pomegranate cultivars a set of four primers (Table 2) have been selected for the analysis.

Amplifications were performed according to the procedure of Williams *et al*. [23]. The reaction volume (25 µL) contained 25 ng of genomic DNA (~1 µl), 2.5 µL of Taq DNA polymerase buffer (10 ×), 100 mM each of dATP, dCTP, dGTP and dTTP, 0.2 pM primer and 1.5 U of Taq DNA polymerase (QBiogene, France). The reaction mixture was overlaid with a drop of mineral oil to avoid evaporation during PCR cycling. Amplification was conducted in a DNA thermal reactor (Crocodile III, QBiogene, France) programmed as followed: 1 cycle of 5 min as a pre-denaturing step at 94 °C followed by 35 cycles each one is composed of a 30s denaturation at 94 °C, 1 min primer annealing at 35 °C and 1 min polymerization at 72 °C with a final extension at 72 °C for 5 min. The amplification products were electrophorised in 1.5% agarose gel containing ethidium bromide (0.5 mg mL^{-1}) in 0.5 × TBE and visualized under UV light transilluminator and photographed. Molecular weights of amplified products were estimated using 1 Kb Ladder (GIBCO-BRL, France). PCR-RAPD assays were performed in duplicate.

3.4. Data Analysis

The DNA profiles were manually scored directly from gel photographs and only repetitive bands, *i.e*., those occurring in the two duplicates, were considered. The fragments produced by each primer were treated as characters and numbered sequentially. Genotypes were scored for the presence (1) or absence (0) of all polymorphic bands. The ability of each primer to differentiate between genotypes was assessed by the estimation of the resolving power (Rp). Rp was calculated according to the formula of Gilbert *et al*. [24] expressed as followed: $Rp = \Sigma\ Ib$; and $Ib = 1-2\ |\ 0.5 - p\ |$, where p represents the proportion of the cultivars having the band I. Besides, the RAPD banding patterns transformed into a binary matrix. A genetic distance matrix was estimated using the Genedist (version 3.572c) program based on the formula developed by Nei and Li [25]. Cluster analysis was made using the Unweighted PairGroup Method with Arithmetic Averages (UPGMA) by PHYLIP software (Phylogeny Inference Package, version 3.5c) [26] and TreeView (Win32, version1.5.2) [27].

4. Conclusions

The present study constitutes a prerequisite for the development of a molecular method suitable in the pomegranate genetic polymorphisms surveying due to the relatively low number of cultivars studied and primers tested. Thus, using the designed method, investigation including a large number either of ecotypes or primers would provide inferences about the genetic diversity structure of Tunisian pomegranate ecotypes. Work is currently in progress in order either gain a deeper insight into the genetic diversity and to molecularly characterise the Tunisian pomegranate germplasm or to enhance its cultivation throughout the establishment of selection programs.

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