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DNA variation and polymorphism in Tunisian plum species (*Prunus* spp): contribution of flow cytometry and molecular markers

H. Ben Tamarzizt[1], D. Walker[2], S. Ben Mustapha[1], D. Abdallah[1], G. Baraket[1], A. Salhi Hannachi[1] and S. Zehdi Azzouzi[1]

[1]Laboratory of Molecular Genetics, Immunology & Biotechnology, Faculty of Sciences of Tunis, University of Tunis El Manar, Campus University, El Manar, Tunis, Tunisia
[2]Departamento de Recursos Naturales, Instituto Murciano de Investigación y Desarrrollo Agrario y Alimentario (IMIDA), Estación Sericícola, Calle Mayor s/n, La Alberca, Murcia, Spain

Corresponding author: A. Salhi Hannachi
E-mail: Amel.SalhiHannachi@fsb.rnu.tn

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ABSTRACT. Plums (*Prunus* spp) are among the most important stone fruit crops in the world. European (*Prunus domestica*) and Japanese (*Prunus salicina*) plums are characterized by different levels of ploidy. Because genetic variability is the prerequisite for any plant-breeding program, we aimed to establish the taxonomic status of Tunisian plums and study their genetic variability. The nuclear DNA content of 45 wild and cultivated Tunisian plums was determined by flow cytometry. Two arbitrary primers (AD10, AD17) were used to elaborate SCAR markers useful to identify plum species. Three wild trees, Zenou 1, Zenou 6, and Zenou 3, which had 2C nuclear DNA contents of 1.99, 2.05, and 2.13 pg, were shown to be hexaploid ($2n = 6x = 48$), whereas the others were diploid ($2n = 2x = 16$). These results suggest that the three hexaploid wild plums

belong to *Prunus insititia*, and the others belong to *Prunus salicina*. No SCAR markers were revealed using the AD10 and AD17 RAPD primers in relation to the ploidy of plums. We note also that AD17 primer appears to be the most informative concerning the genetic diversity. Morphological and pomological traits revealed similarity between introduced and Tunisian plum cultivars. Despite the significant morphological differences found, all the cultivars studied belong to *P. salicina*. The information obtained in this analysis provided on local plum genetic resources will be helpful to establish a core collection, to evaluate genetic diversity, and to initiate an improvement and selection program.

Key words: Flow cytometry; Plum; *Prunus insititia*; *Prunus salicina*; Molecular markers; RAPD

INTRODUCTION

Prunus is a large and diverse genus of plants that belongs to the subfamily Prunoideae of the family Rosaceae (Rehder, 1940).The genus *Prunus* comprises about 400 species of trees and shrubs that produce drupes as fruits, commonly called “stone fruits” It is mainly found in temperate regions in both the northern and southern hemispheres, and it constitutes the third most economically important group of plants in the temperate regions of the world. The large number of *Prunus* species and the frequent interspecific hybridization make the systematic classification in *Prunus* controversial (Dosba et al., 1994). Plum species occur at three levels of ploidy: diploid, tetraploid, and hexaploid. *Prunus domestica* L. (6x), which is one of the European plums, is thought to be derived from a natural cross between *Prunus spinosa* L. (4x) and *Prunus cerasifera* Ehrh (2x). The term ‘Japanese plum’ was originally appliedto *Prunus salicina* Lindl. (2x). The wild species in the *Prunus* genus constitute an important genetic resource and include species that are used medicinally, as rootstocks, as ornamentals, or for food (Pandey et al., 2008). The introduction of promising cultivars of different species of *Prunus* and their subsequent selection to fit agro-climatic regions have allowed considerable diversity to develop in major cultivated species, but this also leads to the evolution of new species and varieties and the extinction of local ones. In addition, the introduction of genes from related species through inter-specific hybridization has been used in several breeding programs throughout the world in order to develop better-adapted cultivars and rootstocks. Rootstock breeding programs that use inter-specific hybridization have introduced useful traits, including size control, adaptation to new environments, and pest resistance, thus producing numerous new varieties (Martinez-Gomez et al., 2003). Nevertheless, breeding barriers exist among taxa that possess different ploidy levels, even within the same section like the section *Prunophora of the Prunus genus*, but hybrids are generally successful when both parents have the same ploidy level (Okie and Weinberger, 1996). In fact, many cultivated genotypes result from cross-pollination making the systematic classification of numerous cultivars extremely difficult. Hence, knowledge of the taxonomic level is important to identify and recognize the gene pool of plum species. Hybridization can induce rapid genomic changes and subsequent changes in the DNA content (Baack et al., 2005). Hence, in recent years, many molecular studies have been established with the aim of identifying and characterizing plum species. Moreover, since tree fruit cultivars are maintained by vegetative propagation, accurate identification of vegetative material is

crucial for nurserymen and growers, and is needed for plant breeder's rights (Goulao et al., 2001). Therefore, molecular markers, such as restriction fragment length polymorphism (RFLP) (Quarta et al., 1996), random amplified polymorphic DNA (RAPD) (Gregor et al., 1994; Warburton and Bliss, 1996; Ortiz et al., 1997; Bellini et al., 1998; Shimada et al., 1998; Casas et al., 1999; Lisek et al., 2007; Li et al., 2007; Ben Tamarzizt et al., 2009), inter simple sequence repeat (ISSR) (Yilmaz et al., 2009), simple sequence reapet SSR (Mnejja et al., 2004; Baraének et al.,2006; Bouhadida et al., 2009), and amplified fragment length polymorphism (AFLP) (Ilgin et al., 2009), and sequences of non-coding region of chloroplast DNA (Ben Mustapha et al., 2013) have been tested. Knowledge of chromosome number and ploidy level is important, especially in plant families and genera where hybridization between species with different chromosome number or ploidy level occurs frequently.

To clarify the taxonomic status of plums in Tunisia, we investigated the Tunisian germplasm by means of DNA quantification using flow cytometry, RAPD markers generated by AD10 and AD17 primers as suggested by Ortiz et al. (1997) to advance their collection, management, and rational utilization. Flow cytometry constitutes a convenient technique that can be used to study ploidy levels, by estimating the nuclear DNA content (Dolezel et al., 2007), and RAPD markers have proven to be a reliable and useful molecular marker for genetic fingerprinting. The specific aims of this study were to 1) identify the taxonomical status of Tunisian plums, 2) to detect duplicated or mislabeled accessions, 3) to evaluate diversity in order to facilitate its use in breeding and in developing a collection strategy, and 4) to analyze the genetic relationship of plum species, focusing on the origin of local resources.

MATERIAL AND METHODS

Plant materials

Forty-five accessions were considered, which represented plum species and their wild relatives. All samples were collected from several localities in northern Tunisia (Ras Jebel, Rafraf, El Alia, Sounine, Ghar El Melh, Douar Hamouda, Bejou, Cap bon, Thibar, Djebba, and Kairouan) (Table1).

Genomic DNA extraction

Total cellular DNA was purified from young frozen leaves according to two procedures: Bernatzky and Tanksley (1986) and a modified procedure as described by Ahrens and Seemüller (1992). The DNA quality was examined by electrophoresis on 0.8% agarose gels, as described by Sambrook et al.(1989), and the DNA concentration was quantified spectrophtometrically.

Flow cytometry procedure

Estimation of nuclear DNA content was performed with a Partec PA II flow cytometer (Partec GMBH, Münster, Germany). The method was based on the protocol described by Bukhari (1997). Samples of growing leaf tissue of *Prunus* and soya were prepared together. Soya has a 2C nuclear DNA content of 2.50 pg. Leaf material was chopped with a razor blade for 30-60 s, in a 60-mm plastic Petri dish containing 0.4 mL extraction buffer (Cystain PI absolute P, Partec GMBH), to which polyvinylpyrrolidone-10 (2.5% w/v), ascorbic acid (12 mM), dithiothreitol (9 mM), and Triton X-100 detergent (0.25% v/v) had been added. The resulting extract was passed through a 30-mL

filter into a 15-mL centrifuge tube. The Petri dish was washed twice with 0.8 mL extraction buffer and the samples were thenfiltered into the 15-mL tube. After centrifugation at 1.100 g for 10 min, the supernatant was removed and the pellet was re-suspended in 1.6 mL Cystain PI absolute P staining buffer (Partec GMBH) to which propidium iodide and RNase had been added (final concentrations of 50 and 17.5 µg/mL, respectively). All stages of the extraction were performed at 4°C. The samples were kept in the dark for 15 min at 37°C, before being filtered through a 30-mL filter. The linearity of the cytometer fluorescence scale was checked regularly using propidium iodide-stained calibration beads (Partec GMBH). At least 5000 nuclei were analyzed in each sample. The equivalent number of base pairs was calculated assuming that 1 pg DNA = 978 Mbp (Dolezel et al., 2007; Greilhuber et al., 2007). One-way ANOVA was performed using SAS (1990), version 6.12. The mean nuclear DNA content was tested by the Student-Newman-Keler test (5%), using SPSS v.11.0.

Table 1. Tunisian plums studied and their locality of origin.

Cultivar	Locality
Bedri1	Ras jebel
Japonia safra	Rafraf
Janha	Rafraf
Ain kounoulia	Rafraf
Cidre1	Rafraf
Adham hmém	Rafraf
Neb zarouk	Rafraf
Hamda	Rafraf
Aouina hamra badri	Rafraf
Ain thaer noman	Rafraf
Golden Japan	El Alia
Ain torkia	Rafraf
Santa Rosa1	Rafraf
Aouina safra morra	Rafraf
Zaghwénia	Rafraf
Ain tasstouria	Sounine
Ain ben moussa	Rafraf
Baydha arbi	Rafraf
Meski kahla1	Rafraf
Meski safra1	Rafraf
Meski kahla2	Rafraf
Bedri2	Ras jebel
Bedri hamra1	Capbon
Black Gold	Kairouan
Bedri hamra2	Kairouan
Bedri hamra3	Kairouan
Bedri hamra4	Kairouan
Stanley	Capbon
Golden Japan2	Capbon
Sauvage1	Thibar
Chaaraouiya	Ghar el melh
Zenou5	Douar Hamouda
Zenou1	Douar Hamouda
Cidre 2	Ghar el melh
Jelya1	Bejou
Golden Japan3	Ghar el meleh
Zenou6	Douar hamouda
Zenou3	Douar Hamouda
Jelya2	Bejou
Zenou7	Douar Hamouda
Sauvage3	Djebba
Black diamond	Capbon
Sandid	Rafraf
Safra jridi	Rafraf
Santa Rosa2	Capbon

RAPD analysis

Two RAPD primers were tested: AD10 (AAGAGGCCAG) and AD17 (GGCAAACCCT) according to Ortiz et al. (1997), these primers produce specific patterns for diploid and hexaploid species. The reactions were carried out in a 25 µL volume reaction mixture containing 20 ng total cellular DNA, 50 pM primer, 2.5 µLTaq DNA polymerase buffer, 1.5 U Taq DNA polymerase (QBIOgéne, France), and 200 mM dNTP (DNA polymerization mix, Pharmacia). The PCRs were then performed in a DNA thermocycler (TC 512, TECHNE) programmed to execute the following cycles: reaction mixtures were heated at 94°C for 5 min as an initial denaturation step before entering 35 cycles, each composed of 30 s at 94°C, 1 min at 35°C, 1 min at 72°C, and a final step of 5 min at 72°C. To reduce the possibility of cross contamination in the amplifications, reaction mixtures without DNA were used as negative controls. Only reproducible products were taken into account for further data analysis. The products of amplification were separated on 1.5% agarose gel at 100 mV for 2 h in 0.5X TBE buffer and detected after ethidium bromide staining according to the method described by Sambrook et al. (1989). To confirm the results, acrylamide gels (10%) were also used.

Data analysis

Polymorphic RAPD bands were scored as present (1) or absent (0) across the 45 genotypes for two RAPDs primers as a binary data matrix. The percentage of polymorphic bands (PPB) was estimated and the ability of RAPD tested primers to differentiate between plums was appreciated by the estimation of their resolving power (Rp) (Prevost and Wilkinson, 1999) The Rp has been described by Gilbert et al. (1999) such that: $Rp = \sum Ib$ where: $Ib = 1 - (2 x| 0.5 -p|)$ where p is the accessions proportion containing the I band. The generated binary matrix was computed with the Gendist program (version 3.572c), using the computer program PHYLIP (phylogeny inference package, version 3.5c) (Felsenstein 1995), producing a genetic distance matrix according to the formula described by Nei and Li (1979). The neighbor program produces a tree-file using the unweighted pair group method with the arithmetic averaging (UPGMA) algorithm.

RESULTS

DNA quantification

Quantification of DNA by flow cytometry (Table 2, Figure 1) suggests that genome size of the Tunisian plum cultivars studies varies significantly. Of the 45 accessions studied, three wild trees ‘Zenou 1’, ‘Zenou 6’, and ‘Zenou 3’, which had 2C nuclear DNA contents of 1.99, 2.05, and 2.13 pg, respectively, were shown to be hexaploid ($2n = 6x = 48$), whilst the others (0.44-0.97 pg DNA) were diploid ($2n = 2x = 16$). The wild tree ‘Zenou1’ contained the highest number of bases (2083.1 Mbp) and cultivar ‘Hamda’ contained the lowest, 432 Mbp (Table 3). The results also show that the ploidy of the introduced cultivars: ‘Black gold’; ‘Black diamond’; ‘Golden Japan’, and ‘Santa Rosa’ were diploid and included in *P. salicina.*

Molecular markers

Here we used two RAPD primers that were suggested by Ortiz et al. (1997) to be able

to differentiate between diploid and hexaploid plum and generate specific SCAR markers that are useful to define the taxonomy statute of Tunisian plum species. The results of the RAPD analysis of 54 plum genotypes are given in Table 3 and Figure 2. Molecular polymorphism was revealed, as demonstrated by RAPD patterns and no specific markers were detected in relation to the ploidy level of plums. Nine and 10 RAPD markers were generated by the AD10 and AD17 primers. Percent polymorphic bands were 90 and 100 for AD10 and AD17, respectively. The AD17 primer appears to be the most informative with a resolving power of 6.4 (Rp of AD10 = 5.5).

Table 2. 2C nuclear DNA contents, the equivalent number of base pairs, and the corresponding ploidy for the *Prunus* cultivars studied.

Cultivar	DNA (pg)	DNA (Mbp)	Ploidy
Ain kounoulia	0.626	612	Diploid
Ain torkia	0.672	656	Diploid
Ain thaer noman	0.520	508	Diploid
Ain ben moussa	0.681	666	Diploid
Ain tasstouria	0.459	449	Diploid
Aouina hamra badri	0.457	447	Diploid
Aouina safra morra	0.567	554	Diploid
Adham hmém	0.635	620	Diploid
Baydha arbi	0.683	667	Diploid
Bedri1	0.598	584	Diploid
Bedri2	0.559	547	Diploid
Bedri hamra1	0.483	471	Diploid
Bedri hamra2	0.642	628	Diploid
Bedri hamra3	0.634	619	Diploid
Bedri hamra4	0.730	713	Diploid
Janha	0.691	676	Diploid
Hamda	0.443	432	Diploid
Japonia safra	0.799	781	Diploid
Safra jridi	0.668	653	Diploid
Neb zarouk	0.634	619	Diploid
Sandid	0.614	600	Diploid
Zaghwénia	0.650	636	Diploid
Cidre1	0.578	564	Diploid
Cidre2	0.544	532	Diploid
Meski kahla1	0.616	602	Diploid
Meski kahla2	0.637	623	Diploid
Meski safra1	0.892	872	Diploid
Chaaraouiya	0.555	542	Diploid
Jelya1	0.971	949	Diploid
Jelya2	0.956	934	Diploid
Sauvage1	0.689	674	Diploid
Sauvage3	0.636	622	Diploid
Zenou1	2.130	2083	Hexaploid
Zenou3	1.995	1951	Hexaploid
Zenou5	0.539	527	Diploid
Zenou6	2.050	2005	Hexaploid
Zenou7	0.699	684	Diploid
Black Gold	0.517	505	Diploid
Black diamond	0.624	609	Diploid
Stanley	0.615	601	Diploid
Golden Japan1	0.587	574	Diploid
Golden Japan2	0.765	747	Diploid
Golden Japan3	0.715	699	Diploid
Santa Rosa1	0.551	538	Diploid
Santa Rosa2	0.699	683	Diploid

Following ANOVA ($P < 0.001$ for the effect of "cultivar"), the LSD values obtained ($P < 0.05$) were 0.184 pg for the nuclear DNA content and 180 Mbp for the equivalent number of base pairs.

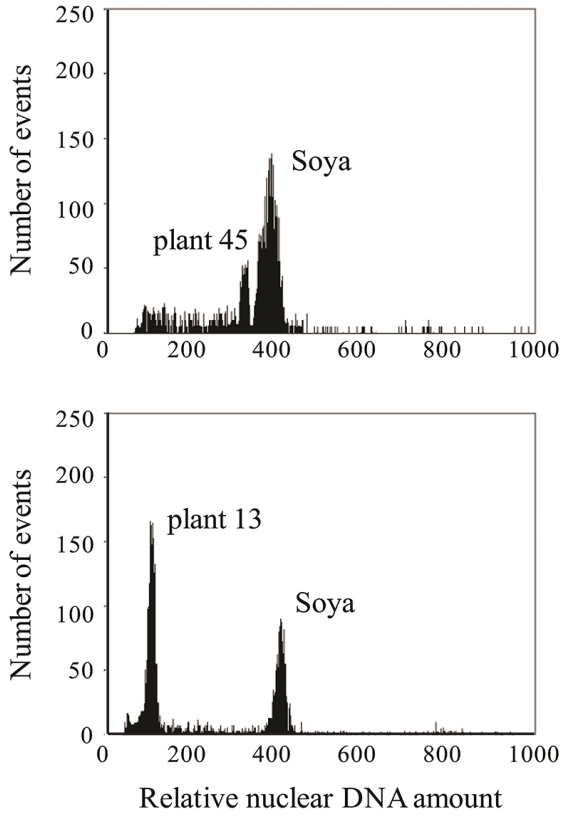

Figure 1. Histogram of the relative nuclear DNA content of *Prunus* plants 13 (*Prunus salicina*; cultivar Ain torkia) and 45 (*Prunus insititia*; cultivar Zenou6), determined by flow cytometry analysis of propidium iodide-stained nuclei with soya (*Glycine max*; 2C nuclear DNA content 2.50 pg) as an internal standard. Nuclei of *Prunus* and soya leaves were isolated, stained, and analyzed simultaneously.

The genetic distances (Nei and Li, 1979) ranged from 0.00 to 1.33 with a mean of 0.53, which suggest a high level of polymorphism at the genomic DNA level of the studied accessions. The lowest distance value (0.00) was observed between [‘Ain kounouliya’ and ‘Adham hmem’]; [‘Ain kounouliya’ and ‘Golden Japan1’]; [‘Cidre1’ and ‘Golden Japan1’]; [‘Meski kahla1’ and ‘Bedri hamra3’]; [‘Meski safra1’ and ‘Bedri hamra2’] cultivars, which seem to be closely related. While the highest distance of 1.33 was calculated between [‘Neb zarrouk’ and ‘Meski safra1’]; [‘Meski kahla1’ and ‘Sauvage1’]; [‘Bedri1’ and ‘Sauvage1’]; [‘Ain torkia’ and ’Safra jridi’]; [‘Golden Japan2’ and ‘Safra jridi’] accessions, suggesting their divergence. Topology of the UPGMA dendrogram (Figure 3) shows that varieties can be classified into two main clusters, the first one labeled (I) is subdivided into two subgroups (Ia and Ib), which contain wild and introduced cultivars, respectively. The second group is divided into two major subgroups (IIa and IIb), which contain the remaining accessions analyzed. Some cultivars presented an important similarity [‘Meski safra1’ and ‘Meski kahla2’]; [‘Meki kahla1’ and ‘Bedri2]; [‘Ain taher noman’ and ‘Ain kounouliya’ ‘Cidre1’]; [‘Sandid’ and ‘Safra jridi’] despite their appellation; these may be explained by misidentification or homonymy problems.

Table 3. Random amplified polymorphic DNA (RAPD) markers generated by AD10 and AD17 primers.

Cultivar	AD10	AD17	
	1140 bp	890 bp	520 bp
Ain kounoulia	-	-	-
Ain torkia	-	+	+
Ain thaer noman	-	-	-
Ain ben moussa	+	+	-
Ain tasstouria	+	+	+
Aouina hamra badri	+	+	-
Aouina safra morra	+	-	-
Adham hmém	+	+	-
Baydha arbi	+	-	-
Bedri1	+	-	-
Bedri2	+	-	-
Bedri hamra1	-	+	-
Bedri hamra2	-	+	-
Bedri hamra3	+	+	-
Bedri hamra4	+	+	-
Janha	-	+	-
Hamda	+	+	-
Japonia safra	-	+	+
Safra jridi	+	+	-
Neb zarouk	-	+	-
Sandid	-	-	-
Zaghwénia	+	-	+
Cidre1	-	+	-
Cidre2	-	-	-
Meski kahla1	+	+	-
Meski kahla2	+	+	-
Meski safra1	+	+	-
Chaaraouiya	+	+	-
Jelya1	+	+	+
Jelya2	+	-	-
Sauvage1	+	+	-
Sauvage3	+	+	-
Zenou1	+	+	-
Zenou3	+	+	-
Zenou5	+	-	-
Zenou6	+	+	-
Zenou7	+	-	-
Black Gold	-	+	-
Black diamond	+	+	-
Stanley	-	-	-
Golden Japan1	+	-	-
Golden Japan2	+	+	-
Golden Japan3	+	+	-
Santa Rosa1	+	-	-
Santa Rosa2	-	-	-

+: presence; -: absence of bands.

DISCUSSION

Little is known about the taxonomic status of the Tunisian plum germplasm.Results of DNA quantification suggest that the three hexaploid wild plums belong to the species *Prunus insititia* and all the other cultivars to *Prunus salicina*, taking into account their diploidy. The wild tree ‘Zenou1’, which belongs to *P. insititia,* had the highest number of bases (2083.1 Mbp) and cultivar ‘Hamda’ (*P. salicina*) had the lowest number of bases (432 Mbp). The results also confirm the ploidy of the introduced cultivars: ‘Black gold’; ‘Black diamond’; ‘Golden Japan’, and ‘Santa Rosa’ were diploid and included in *P. salicina*,as suggested by Goulao et al. (2001). The cultivar ‘Stanley’ was thought to belong to the hexaploid species *P. domestica*; however, flow cytometry showed that

this cultivar is diploid and must be included in *P. salicina*. In this case, flow cytometry permitted the detection and resolution of mislabeling problems. All the remaining cultivars were found to be diploid. In fact, the accessions 'Sauvage1', 'Sauvage3', 'Chaaraouiya', 'Jelya1', and 'Jelya2', which are considered wild cultivars, do not belong to the spontaneous species *P. insititia* or *P. spinosa.* All the other cultivars are diploid and could belong to *P. salicina*. These findings corroborate the work of Mzali et al. (2002) on the basis of morphological characteristics.

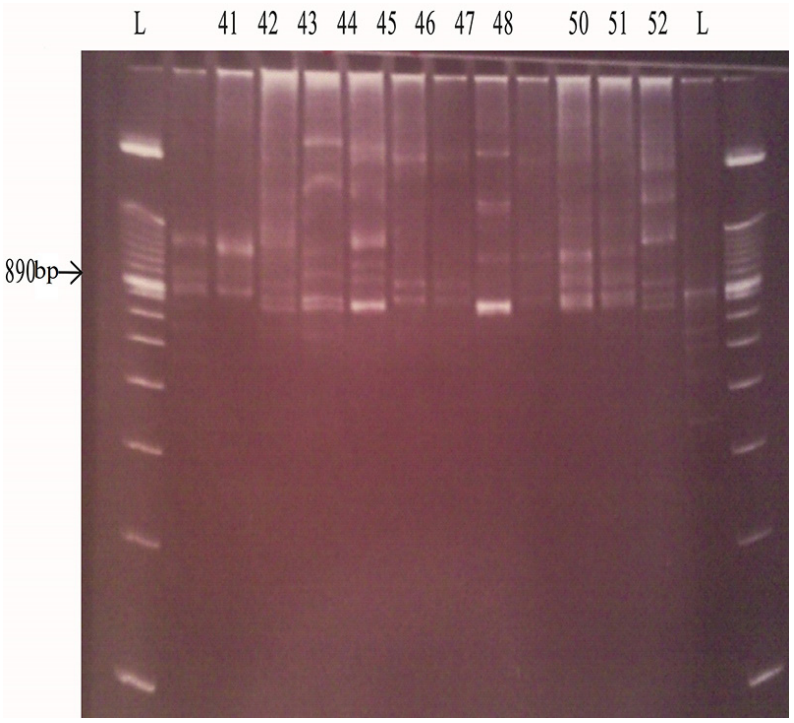

Figure 2. Random amplified polymorphic DNA (RAPD) patterns generated by the AD17 primer. *Lanes*: L = Ladder (100 bp; Invitrogen); 41 = Zenou1; 42 = Cidre2; 43 = Jelya1; 44 = Golden Japan3; 45 = Zenou6; 46 = Zenou3; 47 = Jelya2; 48 = Zenou7; 50 = Sauvage3; 51 = Black diamond; 52 = Sandid.

Polymorphisms at the DNA level have been used in several studies to examine genetic diversity in plums. Previous molecular studies on plums using RAPD markers revealed wide genetic polymorphism among accessions (Gregor et al.,1994; Ortiz et al., 1997; Bellini et al., 1998; Shimada et al., 1999; Lisek et al., 2007; Liu et al., 2007; Ben Tamarzizt et al., 2009), which was explained by the floral biology and different ploidy levels. As demonstrated by Ortiz et al. (1997), use of the arbitrary primers, AD10 and AD17, yields polymorphic amplification products that are specific to the diploid or hexaploid species. In fact, primer AD10 produces a fragment of approximately 1140 bp, which is present only in the Japanese plum. The primer AD17 produces only two patterns, one specifically for the European and the other for the Japanese plum cultivars. These patterns were distinguished by one fragment of approximately 890 bp that is specific to Japanese cultivars and an other amplification product of approximately 520 bp that is characteristic of the European cultivars (Ortiz et al., 1997). Here, we used the AD10 and AD17 primers to reveal the specific markers.

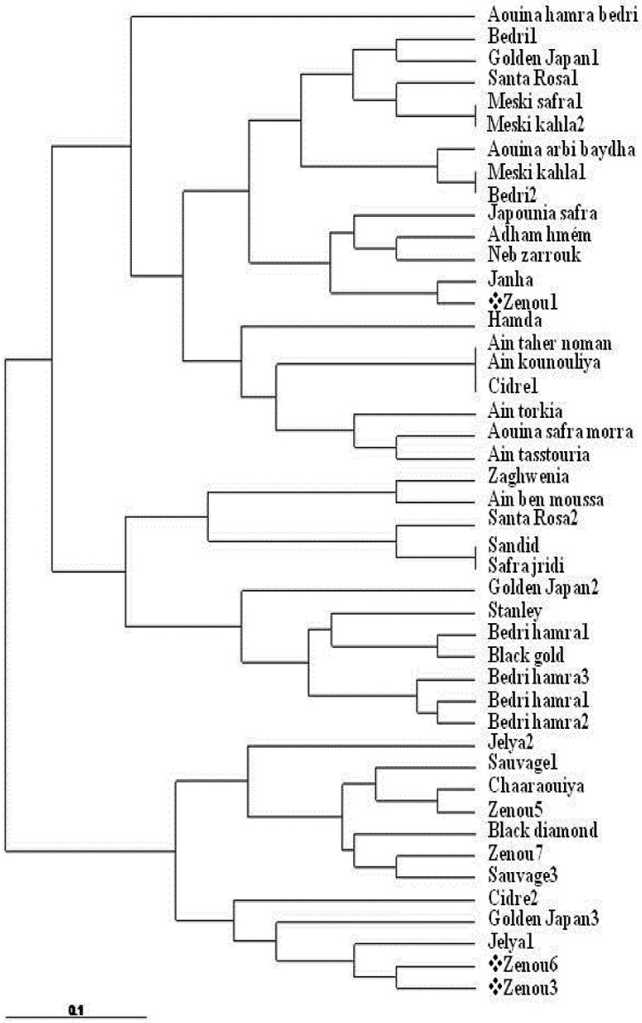

Figure 3. UPGMA dendrogram based on RAPD markers showing the relationships among plum cultivars.

The results reported here are not consistent with those of previous studies investigating RAPD markers as descriptors of the diploid and hexaploid plum species, since the amplified fragments (1140 bp produced by AD10 and 890 bp produced by AD17), which should identify diploid trees (Ortiz et al., 1997), are also amplified in the hexaploid 'Zenou1'; 'Zenou3', and 'Zenou6'. Similarly, the 1140-bp band produced by the AD10 primeris absent in some diploid samples: 'Japounia safra'; 'Janha'; 'Ain kounoulia'; 'Cidre1'; 'Neb zarouk'; 'Ain Taher noman'; 'Ain torkia'; 'Bedri hamra1'; 'Bedri hamra2'; 'Black gold'; 'Stanley'; 'Cidre2', and 'Santa Rosa 2'. Additionally, the AD17 primer that is used to amplify a specific band present in diploids (890 bp) was also tested (Figure 2). This 890-bp fragment was obtained in all cultivars except 'Bedri1'; 'Ain kounoulia', 'Ain taher noman', 'Santa Rosa1', 'Aouina safra morra', 'Ain zaghwénia', 'Aouina arbi baydha', 'Bedri2', 'Stanley', 'Zenou5', 'Zenou7', 'Cidre2', 'Jelya2', 'Sandid', and 'Santa Rosa 2'. These

results contradic those obtained by flow cytometry. Indeed, according to the flow cytometry results, 'Bedri1', 'Ain kounoulia', 'Ain Taher noman', 'Santa Rosa1', 'Aouina safra morra', 'Ain zaghwénia', 'Aouina arbi baydha', 'Bedri2', 'Stanley', 'Zenou5', 'Zenou7', 'Cidre2', 'Jelya2', 'Sandid', and 'Santa Rosa 2' cultivars are diploids. Similarly, the characteristic 520bp fragment, which should be present only in hexaploid plums and is amplified using the AD17 primer (Ortiz et al., 1997), was observed in the diploids 'Japounia safra', 'Ain torkia', 'Ain zaghwénia', 'Ain tasstouria', and 'Jelya1'. These results do not confirm the findings of Ortiz et al. (1997) and the specific RAPD markers do not allow the generation of SCAR markers to recognize plum species. Flow cytometry data reveals that there is intra- and inter-specific DNA variation (Table 3). Trees of the same variety did not present equivalent DNA contents, namely ['Cidre1'; 'Cidre2'], ['Golden Japan1'; 'Golden Japan2'; 'Golden Japan3'], and ['Santa Rosa1'; 'Santa Rosa2']. This variation is also observed among the wild trees ['Zenou1'; 'Zenou3'; 'Zenou5'; 'Zenou6'; 'Zenou7'].

Cluster analysis of plum cultivars revealed a strong distinctness of the genotypes from different geographical regions. As shown by Figure 3, plums are grouped independently of their ploidy level. Cultivar distribution occurs separately from their geographic origins, so typically continuous genetic diversity characterizes local plum germplasm. Additionally, we note that hexaploid cultivars ['Zenou1', 'Zenou3', and 'Zenou6'] do not diverge from diploid cultivars, which confirms the previous results.

Comparison between phenotypic analysis and flow cytometry

Morphological analysis of 20 accessions was performed and important inter-cultivar phenotypic variability was observed by Ben Tamarzizt et al. (2009). Principal component analysis (PCA) was performed using 25 morphological and pomological parameters and showed that there was similarity between the introduced variety 'Santa Rosa' and the local variety 'Cidre1 according to their pomological traits related to fruit and seed characteristics: 'Fruit form', 'Skin color', 'Firmness', 'Juiciness', and 'Acidity'. The introduced cultivars do not differ from the Tunisian ones, indicating the good performance of local cultivars (Ben Tamarzizt et al., 2009). It is important to note that flow cytometry clustered these varieties in the same group of diploid trees, belonging to the Japanese species *P. salicina*. Thus, this cultivar is more important than the introduced one 'Santa Rosa', which have resulted from selection by American breeders such as Luther Burbank since 1883 (Shimada et al., 1999) and Wellington (USA) (Anonymus, 2002; Ben Tamarzizt et al., 2009). Furthermore, significant divergence between local cultivars was observed, especially between "Ain Tasstouria" and "Meski safra 1". In fact, these two Tunisian plums are morphologically distant according to their leaf, branch, fruit, and seed parameters: 'Leaf length', 'Branch length 2006', 'Branch length 2007', 'Fruit length', 'Fruit width', 'Fruit weight', 'Fruit form', 'Skin color', 'Pulp color', 'Firmness', 'Acidity', 'Aroma', 'Sweetness', and 'Seed form. Furthermore, similar varieties, with similar morphological traits, were observed, especially for trees of the same varieties ['Meski kahla 1'; 'Meski kahla 2'] according to their branch, leaf, fruit, and seed parameters. Additionally, the clusters were independent to the geographic origin of the plum cultivars. In this analysis, dispersion of cultivars in the PCA plot appears without any clear aggregation correlated to geographical origin. Despite major differences in morphological appearance, all tested trees have the same ploidy level and belong to the Japanese species *P. salicina.* In fact, many pomological traits were discriminating and characterized each variety. This result can be explained by the environmental adaptation of different varieties.

Morphological study has revealed wide genetic polymorphism among plum accessions, because plums are a very complex group, which includes diploid, tetraploid, and hexaploid species, and because floral biology differs among plum groups (Erturk et al., 2009). The distribution of cultivars occurs independently from their geographic origin. We also note that introduced and Tunisian plums are clustered together in the PCA plot, confirming the efficiency of the local germplasm. These results underline the importance of preserving the genetic resources of plum species since this may enable breeders to select the most diverse genotypes, with interesting fruit characteristics, for crossing and selection programs.

Conflicts of interest

The authors declare no conflict of interest.

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