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Assessment of genetic diversity and relationships among wild and cultivated Tunisian plums (*Prunus* spp) using random amplified microsatellite polymorphism markers

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ABSTRACT. The usefulness of random amplified microsatellite polymorphism markers to study the genetic diversity and relationships among cultivars belonging to *Prunus salicina* and *P. domestica* and their wild relatives (*P. insititia* and *P. spinosa*) was investigated. A total of 226 of 234 bands were polymorphic (96.58%). The 226 random amplified microsatellite polymorphism markers were screened using 15 random amplified polymorphic DNA and inter-simple sequence repeat primers combinations for 54 Tunisian plum accessions. The percentage of polymorphic bands (96.58%), the resolving power of primers values (135.70), and the polymorphic information content demonstrated the efficiency of the primers used in this study. The genetic distances between accessions ranged from 0.18 to 0.79 with a mean of 0.24, suggesting a high level of genetic diversity at the intra- and interspecific levels. The unweighted pair group with arithmetic mean dendrogram

and principal component analysis discriminated cultivars efficiently and illustrated relationships and divergence between spontaneous, locally cultivated, and introduced plum types. These procedures showed continuous variation that occurs independently of the status of the species and geographical origin of the plums. In this study, random amplified microsatellite polymorphism was found to be as a reliable molecular marker for fingerprinting and for examining the diversity study of the plum and its relatives.

Key words: Genetic diversity; *Prunus domestica*; *Prunus insititia*; Random amplified microsatellite polymorphism; *Prunus salicina*; *Prunus spinosa*

INTRODUCTION

The genus *Prunus* (Rosaceae, subfamily Prunoideae) is composed of 5 subgenera, including *Prunophora*, *Amygdalus*, *Cerasus*, *Padus*, and *Laurocerasus* (Badenes and Prafitt, 1995; Aradhya et al., 2004), which all originated mainly in the Northern hemisphere and are widely represented in Europe (Casas et al., 1999). This genus includes nectarine, peach, cherries, plum, apricot, and almond. More than 430 species are distributed mainly in north temperate regions of the world (Pandey et al., 2008). The basic chromosome number for *Prunus* is x = 8. Diploid, tetraploid, and hexaploid plum species are present within the subgenus *Prunophora* (Kester and Asay, 1975; Weinberger, 1975). The European plum (*Prunus domestica* L.), is hexaploid whereas Japanese plum, *Prunus salicina* is diploid (Badenes and Prafitt, 1995). *Prunocerasus* species such as *Prunus americana*, *Prunus hortulana*, and *Prunus rivularis* hybridize naturally, not only with themselves but also with species in the genus *Prunus*, and thus the potential for developing better-adapted varieties is large (Shaw and Small, 2004); however, this makes the botanical classification of the species controversial (Dosba et al., 1994). Several clonal rootstocks for stone fruit species have been released (Moreno et al., 1995a,b), and some are currently under extensive commercial exploitation. Desirable quality traits in the species have been selected (Salesses et al., 1994), including resistance to root knot nematode (*Meloidogyne* spp) (Scotto La Massèse et al., 1984). Plum characterization is of particular interest because the species has been domesticated independently on 3 different continents (Europe, America, and Japan) (Nassi et al., 2003) because of their adaptation to many ecogeographic conditions. Genetic erosion, resulting from the loss of numerous precious varieties, was increased through cultural practices as monovarietal culture and the extension of plant diseases such as coryneum caused by the fungus *Coryneum beijerinckii* or virus diseases caused by 2 ilarviruses, *Prunus* necrotic ringspot virus and prune dwarf virus, which infect plum orchards (Boulila and Marrakchi, 2001).

In Tunisia, plums (*Prunus* spp), characterized by their large adaptive capacities to numerous environments and soils, grow in northern Tunisian. Cultivated for their edible fruits, these plants are preferentially propagated using the grating method. This tree constitutes an important fruit crop and includes several elite cultivars. The local genetic resources of plum are important in the fruit industry and can be consumed fresh or dried. Unfortunately, as occurs in many other crop plants, the genetic base of indigenous and wild species of Tunisian plums is being eroded because of habitat destruction, introduction of outside cultivars/varieties, and lack

of appropriate conservation and management strategies. Genetic diversity of plum cultivars has been evaluated through the use of morphological and pomological parameters according to the International Plant Genetic Resources Institute descriptors (IPGRI/CEC, 1984). Different environmental influences can affect these morphological characteristics. Polymorphisms were assessed using molecular markers, and several molecular markers were tested to identify cultivars and describe genetic diversity. Previous studies used biochemical and molecular markers such as isozymes (Chyi and Weeden, 1984) and restriction fragment length polymorphism (RFLP) (Shimada, 1996). Random amplified polymorphic DNA (RAPD)-amplified fragment length polymorphism (AFLP) markers have also been used to examine *Prunus* species not only for genetic diversity analysis but also for establishing genetic maps (Casas et al., 1999; Ben Tamarzizt et al., 2009). Goulão et al. (2001) used AFLPs and inter-simple sequence repeat (ISSR) markers to characterize both diploid and hexaploid plum cultivars. Simple sequence repeats were also established in nuclear and cytoplasmic DNA in Rosaceae species for molecular characterization (Howad et al., 2005; Ohta et al., 2005; Bouhadida et al., 2007). Thus, the aim of this study was to characterize 54 plum cultivars using 15 random amplified microsatellite polymorphism (RAMPO) combinations to evaluate the genetic diversity and to establish relationships among Tunisian plum cultivars. These markers were used to generate genetic fingerprints in plum species. Only a limited number of studies have been conducted to examine the genetic variation of plum species in Tunisia. There have been no reports describing the application of RAMPO markers for determining genetic diversity in *Prunus* species. Our results provide new insights into the evolutionary relationships among these species.

MATERIAL AND METHODS

Plant materials

A set of 54 Tunisian plum accessions, collected throughout Tunisia were analyzed in this study (Table 1, Figure 1). Among these, 7 are introduced varieties belonged to *P. salicina* and *P. domestica* species, 14 belonged to wild-type *Prunus insititia* and *Prunus spinosa*, and 33 were local cultivars (*P. salicina*). Plant material was sampled from young leaves of adult trees and frozen at -20°C for DNA extraction. Cultivated plums represent both the *P. salicina* and *P. domestica* species.

Genomic DNA extraction

Total cellular DNA was purified from young frozen leaves by using 2 procedures, including that described by Bernatzky and Tanksley (1986) and a modified procedure of Ahrens and Seemüller (1992). DNA quality was examined by electrophoresis on 0.8% agarose gels, as described by Sambrook et al. (1989), and DNA concentration was quantified spectrophotometrically.

Primers and polymerase chain reaction (PCR) assays

Primers

In this study, 2 types of primers were used. Initially, DNA amplification was conducted using 5 universal decamer oligonucleotides (OPH-11, OPA-2, OPA-18, OPM-1, and

Table 1. List of the Tunisian plum accessions studied and their origins.

Accession	Geographic origin	Native origin of variety	Species name
Bedri1	Ras Jebel	Local variety	*Prunus salicina* L.
Bedri2	Ras Jebel	Local variety	*Prunus salicina* L.
Jabonia safra	Rafraf	Local variety	*Prunus salicina* L.
Janha	Rafraf	Local variety	*Prunus salicina* L.
Ain kounoulia	Rafraf	Local variety	*Prunus salicina* L.
Ain bagra	Rafraf	Local variety	*Prunus salicina* L.
Cidre1	Rafraf	Local variety	*Prunus salicina* L.
Cidre2	Ghar el Melh	Local variety	*Prunus salicina* L.
Adham hmém	Rafraf	Local variety	*Prunus salicina* L.
Neb zarouk	Rafraf	Local variety	*Prunus salicina* L.
Hamda	Rafraf	Local variety	*Prunus salicina* L.
Sandid	Rafraf	Local variety	*Prunus salicina* L.
Black diamond*	Menzel Bouzelfa	Introduced variety USA	*Prunus salicina* L.
Golden Japan1*	El Alia	Introduced variety USA	*Prunus salicina* L.
Golden Japan2*	Menzel Bouzelfa	Introduced variety USA	*Prunus salicina* L.
Golden Japan3*	Ghar el Melh	Introduced variety USA	*Prunus salicina* L.
Black Golden*	Kairouan	Introduced variety USA	*Prunus salicina* L.
Santa Rosa1*	Rafraf	Introduced variety USA	*Prunus salicina* L.
Santa Rosa2*	Menzel Bouzelfa	Introduced variety USA	*Prunus salicina* L.
Stanley*	Menzel Bouzelfa	Introduced variety USA	*Prunus domestica* L.
Meski hamra1	Rafraf	Local variety	*Prunus salicina* L.
Meski hamra2	Rafraf	Local variety	*Prunus salicina* L.
Meski safra1	Rafraf	Local variety	*Prunus salicina* L.
Meski safra2	Rafraf	Local variety	*Prunus salicina* L.
Meski kahla1	Rafraf	Local variety	*Prunus salicina* L.
Meski kahla2	Rafraf	Local variety	*Prunus salicina* L.
Zaghwénia	Rafraf	Local variety	*Prunus salicina* L.
Ain Tasstouria	Sounine	Local variety	*Prunus salicina* L.
Ain Torkia	Rafraf	Local variety	*Prunus salicina* L.
Ain Ben moussa	Rafraf	Local variety	*Prunus salicina* L.
Ain Taher Noman	Rafraf	Local variety	*Prunus salicina* L.
Bedri hamra1	Menzel Bouzelfa	Local variety	*Prunus salicina* L.
Bedri hamra2	Kairouan	Local variety	*Prunus salicina* L.
Bedri hamra3	Kairouan	Local variety	*Prunus salicina* L.
Bedri hamra4	Kairouan	Local variety	*Prunus salicina* L.
Baydha arbi	Rafraf	Local variety	*Prunus salicina* L.
Aouina safra morra	Rafraf	Local variety	*Prunus salicina* L.
Aouina hamra bedri	Rafraf	Local variety	*Prunus salicina* L.
Aouina safra bedri	Rafraf	Local variety	*Prunus salicina* L.
Safra Jridi	Rafraf	Local variety	*Prunus salicina* L.
Sauvage**	Rafraf	Local origin	*Prunus insititia* L.
Sauvage1**	Thibar	Local origin	*Prunus spinosa* L.
Sauvage2**	Djebba	Local origin	*Prunus spinosa* L.
Sauvage3**	Djebba	Local origin	*Prunus spinosa* L.
Zenou1**	Douar Hamouda	Local origin	*Prunus insititia* L.
Zenou2**	Douar Hamouda	Local origin	*Prunus insititia* L.
Zenou3**	Douar Hamouda	Local origin	*Prunus insititia* L.
Zenou4**	Douar Hamouda	Local origin	*Prunus insititia* L.
Zenou5**	Douar Hamouda	Local origin	*Prunus insititia* L.
Zenou6**	Douar Hamouda	Local origin	*Prunus insititia* L.
Zenou7**	Douar Hamouda	Local origin	*Prunus insititia* L.
Jelya1**	Bejou	Local origin	*Prunus insititia* L.
Jelya2**	Bejou	Local origin	*Prunus insititia* L.
Chaaraouiya**	Ghar el Melh	Local origin	*Prunus insititia* L.

*Introduced varieties; **Wild-type.

OPM-5) purchased from Operon Technology Inc. (Huntsville, AL, USA) used to perform RAPD assays and 3 primers (02, 04, and 05) that were complementary to simple sequence repeats used to perform ISSR assays (Table 2). According to Chatti et al. (2007) and Rhouma et al. (2008), the RAMPO method consists of the combination of 2 PCR steps.

Figure 1. Map of Tunisia. Geographical distribution of the plums analyzed.

Table 2. Characteristics of the primers used in this study.

Primer	Label	Sequence 5'-3'	Tm (°C)
RAPD	OPH-11	CTTCCGCAGT	35°
	OPA-2	TGCCGAGCTG	35°
	OPA-18	AGGTGACCGT	35°
	OPM-1	GTTGGTGGCT	35°
	OPM-5	GGGAACGTGT	35°
ISSR	02	$(AG)_{10}T$	57°
	04	$(CT)_{10}A$	57°
	05	$(CT)_{10}G$	60°

PCR assays

Initially, RAPD-PCRs were carried out in a final volume of 25 µL according to Ben Tamarzizt et al. (2009). ISSR-PCRs were performed in a 25-µL reaction mixture volume containing 2 µL RAPD-PCR products, 120 pg primer, 200 µM of each dNTP (DNA polymerization mix, Pharmacia, Orsay, France), 2.5 µL 10X Taq DNA polymerase buffer, and 1.5 U *Taq* DNA polymerase (Q-Biogéne, Strasbourg, France). Amplifications were carried out in a DNA thermocycler (TC 512, Techne, Cambridge, UK) using the following temperatures cycles: an initial denaturation at 94°C for 5 min, 30 cycles consisting

of 30 s at 94°C for denaturation, 90 s at the corresponding annealing temperature (°C), and 90 s at 72°C for elongation. The last cycle was followed by a final incubation for 5 min at 72°C and the PCR products were stored at 4°C before analysis. Two independent RAMPO reactions were performed for each DNA sample to ensure the reproducibility of the generated banding patterns. Master mixes of the reaction constituents were used to reduce cross-contamination and variation in the amplification reactions. A negative control (reaction mix without DNA) was also included. Amplification of nuclear DNA products was evaluated by electrophoresis on 1.5% agarose gels in 0.5x Tris-Borate-EDTA buffer, pH 8.3, stained using ethidium bromide, and visualized under ultraviolet light (Sambrook et al., 1989). Amplified fragment sizes were estimated with a 1-kb ladder DNA marker (Invitrogen, Carlsbad, CA, USA).

Data analysis

The presence (1) or absence (0) of bands was scored to determine the total number of bands. Only polymorphic bands were taken into account and used to calculate the percentage of polymorphic bands. For each primer combination, the ability to discriminate cultivars was assessed by estimating the resolving power (R*p*) that has been described to correlate strongly with the ability to distinguish between accessions (Prevost and Wilkinson, 1999) according to the formula described by Gilbert et al. (1999):

$$\mathbf{R}p = \sum \mathbf{Ib}, \text{ where } \mathbf{Ib} = 1 - (2 \times |0.5 - p|) \quad \text{(Equation 1)}$$

where *p* is the proportion of accessions containing the band I.

Polymorphic information content (PIC) was estimated using the formula described by Lynch and Walsh (1998):

$$\mathbf{PIC} = 1 - \sum_{i=1}^{k} \mathbf{Pi}^2 \quad \text{(Equation 2}$$

where *k* is the total number of alleles detected for a given marker locus and *Pi* is the frequency of the ith allele in the set of genotypes investigated.

A binary data matrix was obtained and computed using the Genedist program (version 3.572c) with the program PHYLIP (phylogeny inference package, version 3.5c) (Felsenstein, 1995) to produce a genetic distance matrix using the formula described by Nei and Li (1979). To test the usefulness of the overall information provided by the RAMPO data for establishing phylogenetic relationships between plums, cluster analysis was performed using the unweighted pair group with arithmetic mean (UPGMA) method. The UPGMA dendrogram was constructed using PHYLIP (Phylogeny Inference Package, version 3.5c) (Felsenstein, 1995) and the Treeview software of Page 1996 (Win32, version 1.5.2). Principal component analysis (PCA), as a multivariate analysis method, was applied using the XLSTAT program (2009) to determine the distribution and genetic relationships among plum accessions.

RESULTS

Primer resolving power and polymorphism

Fifteen primer combinations generated clear and reproducible bands scored as RAMPO markers (Table 3). A total of 234 RAMPO fragments were consistently resolved and 226 were polymorphic ranging in size from 200 to 2500 base pairs (Figure 2). The number

Table 3. Primer combinations, percentage of polymorphic bands (PPB), resolving power (R*p*), and polymorphism information content (PIC) of the tested primers.

Primer combination	Total No. of bands	Polymorphic bands	PPB (%)	R*p*	PIC
OPH-11x02	16	16	100	9.51	0.81
OPH-11x04	15	13	86.66	6.74	0.85
OPH-11x05	13	11	84.61	5.29	0.87
OPA-2x02	17	17	100	11.74	0.79
OPA-2x04	15	15	100	10.66	0.85
OPA-2x05	21	19	90.47	11.18	0.84
OPA-18x02	20	20	100	12.25	0.85
OPA-18x04	15	15	100	10.03	0.85
OPA-18x05	15	15	100	7.51	0.79
OPM-1x02	14	13	92.85	8.40	0.85
OPM-1x04	14	13	92.85	8.33	0.87
OPM-1x05	10	10	100	6.18	0.69
OPM-5x02	18	18	100	9.29	0.87
OPM-5x04	16	16	100	7.92	0.88
OPM-5x05	15	15	100	10.59	0.75
Total	234	226	-	135.7	-
Average	15.6	15.06	96.49	9.04	0.82

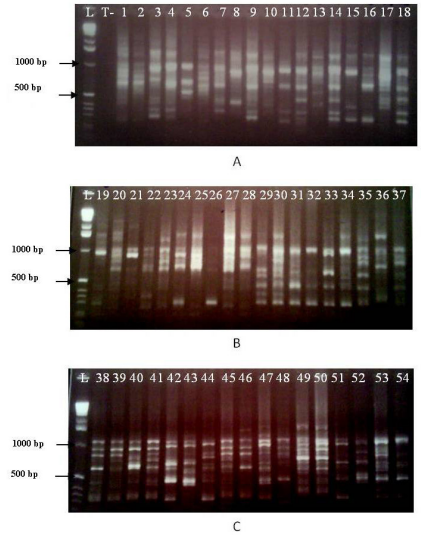

Figure 2. Polymorphism in DNA samples of plums and random amplified microsatellite polymorphism (RAMPO) fingerprints generated by OPA-18x02 (**A**), OPA-18x04 (**B**), and OPA-18x05 (**C**) primer combinations. These primer combinations produced clear and unambiguous bands. *Lane* = ladder (1 kb, Invitrogen); *lane T-* = negative control; *lanes 1-54* = amplification patterns for accessions studied.

of markers varied from 10 (OPM-1x05) to 20 (OPA-18x02), with a mean of 15.06 bands per primer combination. The percentage of polymorphic bands was high and varied from 84.61% for OPH-11x05 to 100% for the OPH-11x02, OPA-2x02, OPA-2x04, OPA-18x02, OPA-18x04, OPA-18x05, OPM-1x05, OPM-5x02, OPM-5x04, and OPM-5x05 primer combinations, with an average of 96.49%. Thus, all primers tested were considered to be sufficiently powerful to detect DNA polymorphisms in plum accessions, as demonstrated by the highest value of *Rp* calculated for the tested primers. Indeed, the *Rp* rate varied from 5.29 (OPH-11x05) to 12.25 (OPA-18x02), with a mean 9.04 (Table 3). In contrast, as shown in Figure 3, the PIC values varied from 0.69 to 0.88, with a mean of 0.82. In fact, 154 of the 226 RAMPOs exhibited PIC values ranging from 0.8 to 1. Thus, the OPA-18x02 combination products showing the higher number of polymorphic bands was the most efficient primer combination for revealing the genetic diversity of plum trees.

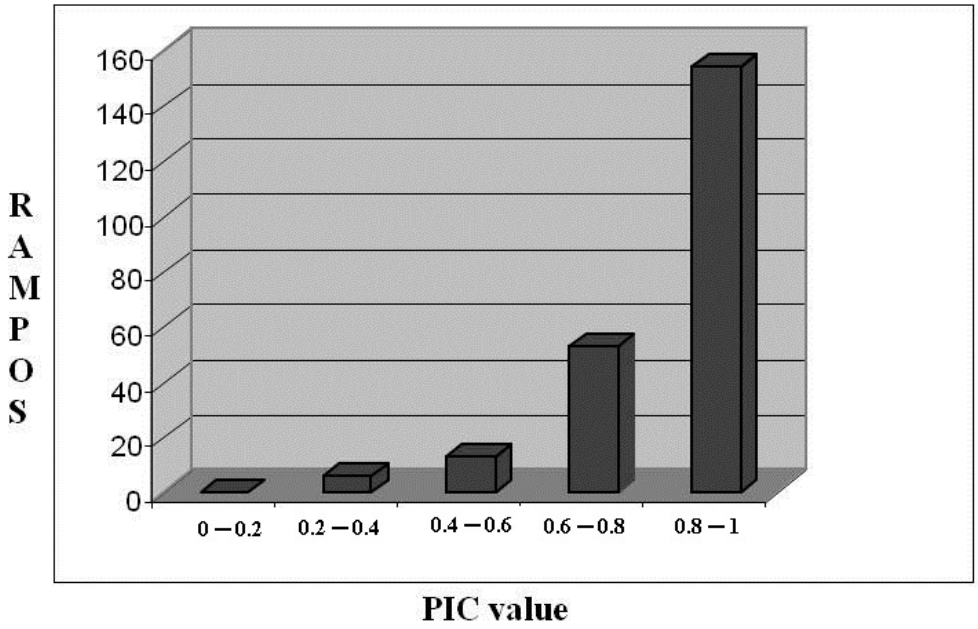

Figure 3. Distribution of random amplified microsatellite polymorphism (RAMPO) markers obtained considering the polymorphism information content (PIC) for plum accessions studied and frequency distribution of PIC range values.

Genetic diversity and phylogenetic relationships

Polymorphic RAMPO bands were scored as present (1) or absent (0) across all 54 genotypes for each primer-pair combination and used as a binary data matrix. Using the Nei and Li (1979) formula, a genetic distance matrix was constructed. The genetic distances ranged from 0.18 to 0.79, with a mean of 0.24, suggesting a high level of polymorphism recorded at the genomic DNA level of the accessions studied. For *Prunus* species, a good range of PIC value was observed (0.8-1), demonstrating the significant genetic diversity among plum accessions.

Intra and interspecific diversity

The lowest distance value of 0.18 was observed between the Bedri hamra2 and Bedri hamra3 cultivars, which appeared to be closely related as they showed the same denomination, suggesting homonymy. The highest distance of 0.79 was calculated between the Golden Japan3 and Zaghwénia accessions, suggesting their divergence.

Cluster analysis of plum cultivars revealed strongly distinct genotypes from different geographical regions. The topology of the UPGMA dendrogram (Figure 4) showed the classification of varieties into 2 main clusters: the first one labeled (a) contained local cultivars

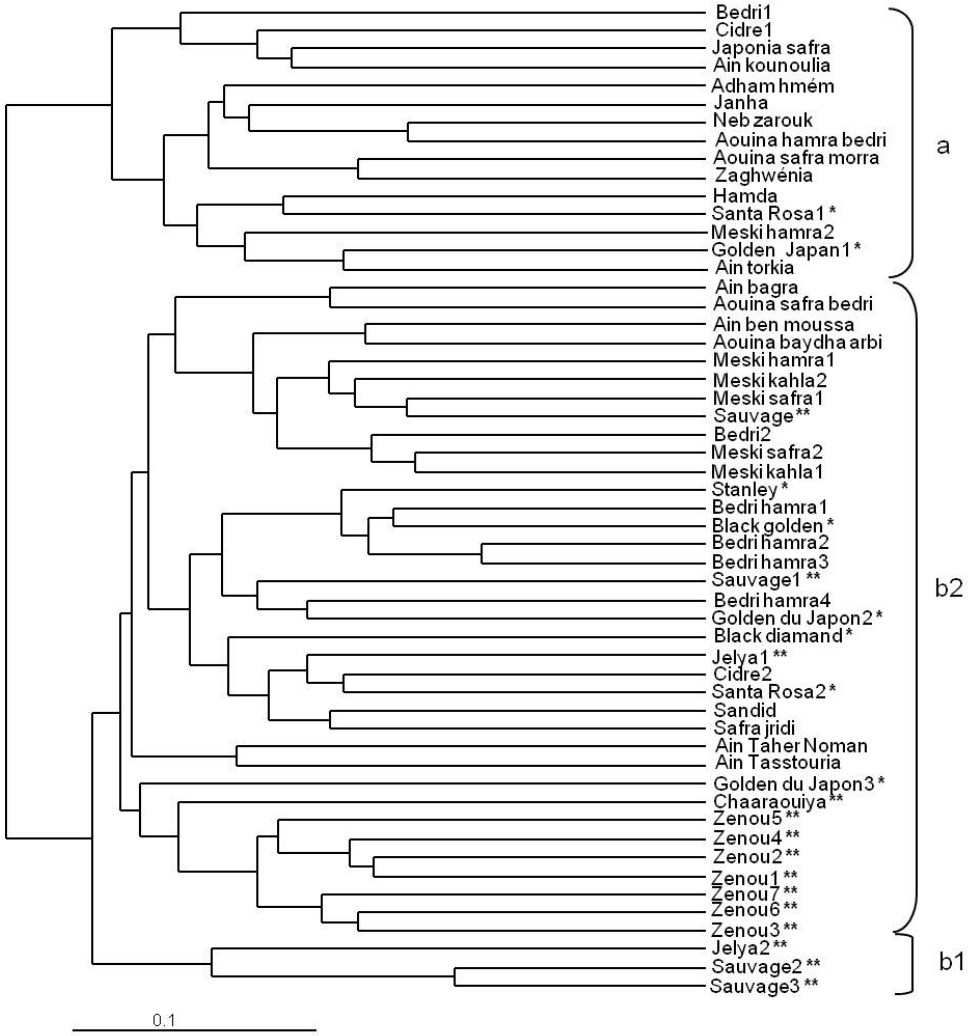

Figure 4. Unweighted pair group method with arithmetic mean (UPGMA) dendrogram illustrating the genetic relationships between 54 Tunisian plum accessions based on random amplified microsatellite polymorphism (RAMPO) markers. *Introduced varieties; **Wild-type (*Prunus insititia* and *Prunus spinosa*).

collected from Rafraf and Ras Jebel: Bedri1, Cidre1, Japonia safra, Ain kounoulia, Adham hmém, Janha, Neb zarouk, Aouina hamra bedri, Aouina safra morra, Zaghwénia, Hamda, Meski hamra2, and Ain torkia and two introduced varieties: Santa Rosa1 and Golden Japan1. The second group was divided into 2 major subgroups, (b1) and (b2), which contained the remaining accessions analyzed from Douar Hamouda, Rafraf, Ras Jebel, Bejou, Ghar el Melh, Sounine, El Alia, Thibar, Djjeba, Cap Bon (North), and Kairouan (Center). The first subgroup (b1), significantly divergent, contained 3 wild genotypes belonging to the *P. insititia* species Sauvage2 and Sauvage3, which originated from Djjeba and Jelya 2 from Bejou. The second subgroup (b2) included cultivars and wild types originating from all prospected locations. Thus, no complete eco-geographical clustering was observed within these subgroups. Typically, continuous genetic diversity characterized local plum germplasm. Additionally, wild cultivars named Zenou were grouped together and clustered with plum cultivars representing the *P. domestica* or *P. salicina* species. However, cultivars with the same appellation were distinguished using RAMPO markers, including Bedri, Meski, Cidre, and Golden Japan (Figure 4 and Figure 5). Our results suggest the presence of intra-varietal and intra-specific diversity. In fact, these plums belonged to the *P. salicina* species. The results of the study are important for accurate identification of elite genotypes in breeding programs.

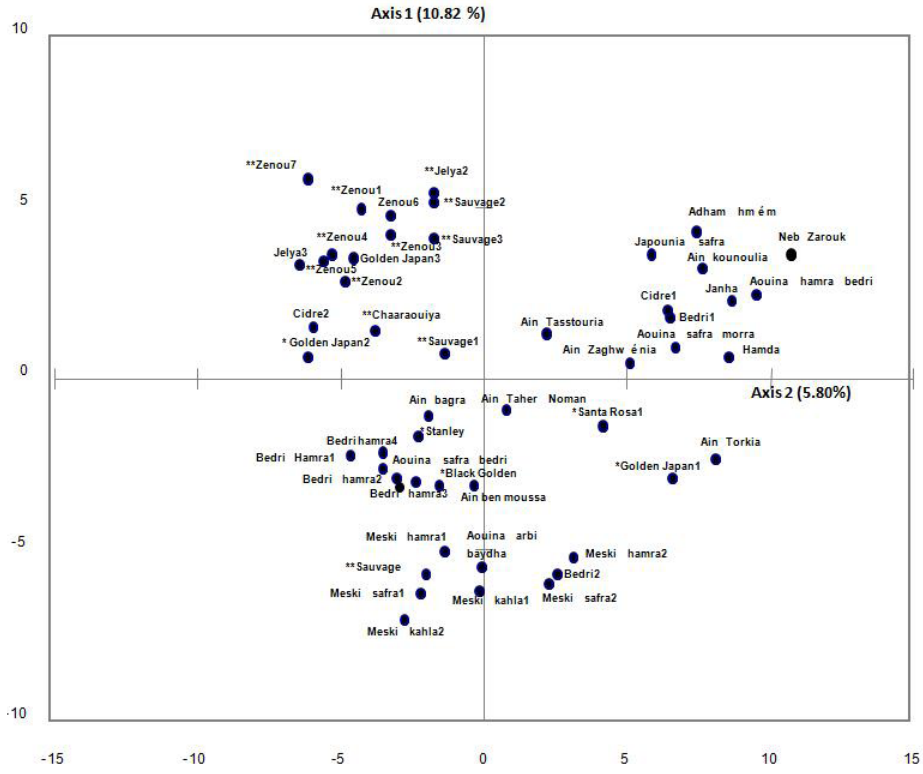

Figure 5. Dispersion of 54 Tunisian plum accessions in the plot (1-2) of the principal component analysis based on 226 random amplified microsatellite polymorphism markers. Local cultivars (*Prunus salicina*); *Introduced varieties (*P. salicina* and *P. domestica*); **Wild-type (*P. insititia* and *P. spinosa*)

Additionally, multivariate PCA based on the RAMPO data (Figure 5) showed similar results as cluster analysis. Groupings of plum genotypes using PCA were based mainly on the first 3 axes, which accounted for 21.76% of the variability observed. These 3 axes accounted for 10.81, 5.80, and 5.140% of the observed diversity, respectively, for PC1, PC2, and PC3. The variables integrated positively by PC1 were markers generated by the primer combinations OPH-11x05, OPA-2x05, and OPM-5x05. This first component was negatively correlated with markers generated by the OPA2x02 combination. The second axis was positively correlated with markers generated by the OPA-2x02, OPA-2x04, OPA-2x05, and OPA-18x05 combinations and negatively with those produced by OPH-11x04, OPM-1x02, and OPM-5x04. Our results suggest that the distribution of cultivars occurs independently from their geographic origin and species status.

Genetic diversity between cultivated and wild-type plums

In the dendrogram, all plum accessions clustered into 2 main groups (a and b). Group a consisted of 15 cultivated plum of *P. salicina*. The other 39 accessions were included in group b (Figure 4). Group b could be divided into 2 subgroups. Subgroup b1 included 3 wild-type species (1 *P. insititia*, 2 *P. spinosa*). The remaining 36 accessions were classified as sub-group b2, which included 11 wild trees representing *P. insititia* and *P. spinosa* and 25 cultivated plum of *P. salicina* and *P. domestica*. PCA was used to efficiently discriminate the cultivars and illustrated the relationships and divergence between spontaneous, locally cultivated, and introduced plum types. Discrimination of spontaneous trees was clearly noted for 3 trees representing the sub-cluster b1. Interestingly, the first axis of PCA permitted the separation of wild-type from cultivated species. Additionally, an important homogeneity between introduced and local accessions was observed, confirming the results of previous studies (Casas et al., 1999; Shimada et al., 1999; Baránek et al., 2006; Ayanoglu et al., 2007; Ben Tamarzizt et al., 2009).

DISCUSSION

In this study, RAMPO markers were used to examine the genetic diversity of cultivated and wild plum accessions as well as to establish relationships between the 2 compartments to understand the origin of Tunisian plum germplasm. A relatively high level of genetic diversity was observed in both the wild and cultivated gene pools.

The genetic diversity of plum cultivars was assessed using a set of 15 primer combinations, which gave rise to 234 bands. Additionally, the high collective R*p* rate obtained (135.7) indicated the efficiency of the primers. In fact, these primers generated 226 polymorphic bands of 234 fragments, with a mean of 15.6 per primer pair. This is significantly higher than values reported in other studies based on RAPD markers in plum cultivars (Casas et al., 1999; Shimada et al., 1999; Baránek et al., 2006) and AFLP markers (Ayanoglu et al., 2007). Similar results were also observed for Tunisian plum shrubs using the RAPD technique (Ben Tamarzizt et al., 2009). Thus, for local plum germplasms, the RAMPO technique produced a larger number of markers compared with other techniques. The genetic distances calculated among the trees indicated high levels of molecular polymorphism. Similar results were obtained for other fruit trees, such as fig by Chatti et al. (2007) and date-palms by Rhouma et al. (2008). RAMPO suggests intravarietal diversity as demonstrated by discrimination of Bedri, Meski,

Cidre, and Golden Japan cultivars. These may be considered as cases of misidentification or homonymy and suggest the presence of polyclonal varieties. These results agree with those of previous studies using RAPD markers such as a study conducted by Ben Tamarzizt et al. (2009). A common genetic basis characterizes the genotypes studied, despite of their morphological divergence and taxonomic status (Ben Tamarzizt et al., 2009).

UPGMA cluster analysis showed the presence of 2 major groups. The plum repartition occurred independently of the geographic origin of trees. Additionally, some wild trees were not differentiated from local plum, except Zenou1, Zenou2, Zenou3, Zenou4, Zenou5, Zenou6, and Zenou7 and Sauvage2 and Sauvage3, which had the same origins as Douar Hamouda and Djebba, respectively. However, the classification of cultivated (*P. domestica* and *P. salicina*) and wild shrubs (*P. insititia* and *P. spinosa*) obtained using the UPGMA dendrogram and PCA did not show strong discrimination between the 2, indicating a unique origin. There was no distinction between Tunisian accessions and introduced samples characterized by powerful pomological and agronomical traits to demonstrate the performance of local cultivars. PCA showed a similar result, confirming those found using cluster analysis. In fact, we note the association between wild plums that were separated according to the first axis and other cultivars.

Similar results were reported by Ben Tamarzizt et al. (2009). This analysis demonstrated that cultivars clustered jointly, and those such as Santa Rosa1 and Santa Rosa2 and Golden Japan and Golden Japan2 belong to *P. salicina* (diploid 2n = 16); additionally, those grouped with cultivars Black Golden and Black diamond clustered with the Stanley cultivar, representing *P. domestica* (hexaploid 2n = 48). As demonstrated by Shimada et al. (1999), RAPD markers can be used to differentiate between the "Japanese plum group" *P. salicina* and the "European plum group" *P. domestica*. Thus, continuous genetic diversity characterizes plum species cultivars. It is widely thought that the hexaploid European plums *P. domestica* and *P. insititia* arose from a cross between a diploid (2n = 2x = 16) cherry plum or myrobalan, *Prunus cerasifera*, and a tetraploid (2n = 4x = 32) sloe or blackthorn, *P. spinosa* (Crane and Lawrence, 1952). However, another hypothesis based on RFLP variation in cpDNA genes suggested that the European plum might have originated from polyploid forms of the myrobalan plum (Reynders and Salesses, 1991). Both hypotheses agree with our findings and the levels of genetic similarities among diploid and hexaploid plums. Despite their economic and genetic values, no detailed study has been conducted to evaluate the extent and pattern of genetic diversity within plum species in its native distributional range. The only available information regarding these important plant genetic resources pertains to molecular (RAPD) and a morphological characterization (Ben Tamarzizt et al., 2009).

Detailed characterization and evaluation, including molecular analysis of genetic diversity, therefore, is needed to assess the conservation requirements and utilitarian prospects of plum species. Assessment of genetic diversity using morphological markers alone has serious limitations, particularly in species of a complex genus such as *Prunus*, whose taxonomy is otherwise in a chaotic state because of frequent incidences of hybridization and polyploidy. RAMPO markers revealed the narrow genetic diversity on which domesticated accessions are based by clustering a set of cultivars and wild types together. The results showed that molecular markers are useful for studying intra- and inter specific genetic diversity in plums and describing the diversity and hybridization events within plum gene pools during their history. Plum has complicated ploidy levels and show different parentages in natural hybridization between species. Natural hybridization and introgression occur widely in plants and play important roles in their evolution (Jarvis and Hodgkin, 1999). Hybridization is of great interest in

plant evolutionary studies because it generates new genotypes, thus, increasing genetic polymorphism, which may lead to new adaptations (Rieseberg, 1991) and the formation of new ecotypes, or species, as suggested by Rieseberg (1997) and Soltis and Soltis (1999). These new combinations of genes resulting from hybridization and introgression between wild relatives and their crop cultivars are important in the evolution of domesticated crop species (Jarvis and Hodgkin, 1999).

The RAMPO technique often reveals a much larger number of polymorphic markers for assessing genetic diversity in plum cultivar identification, in the management of germplasm resources, and for molecular differentiation of plum germplasm resources of Tunisia. In this study, we conducted extensive sampling from the plum natural habitat to assess RAMPO and genetic variability in the species *P. domestica* and *P. salicina* as well as the closely related wild species *P. insititia* and *P. spinosa* present in the northern regions of Tunisia. In conclusion, RAMPO markers can be successfully used to determine the level and structure of plum genetic diversity. The relationships among cultivated and spontaneous genotypes studied from different geographical regions may reveal the proximity between the two gene pools and indicate the relatively of genetic differentiation between the genotypes, which may have resulted from an inherently narrow genetic base from which the plum was domesticated, combined with historical migration of germplasm, and natural hybridization.

CONCLUSIONS

This is the first study examining the characterization of *Prunus* species based on RAMPO markers as a molecular markers. We found that the molecular markers could be used to study genetic diversity and distribution within and among a set *Prunus* species (*P. salicina*, *P. domestica*, *P. insititia*, and *P. spinosa*). This study revealed the rich genetic diversity among plum accessions and indicated that RAMPO markers can be used to identify fruit resources and to explain their genetic diversity. RAMPO markers revealed the narrow genetic diversity among wild (*P. insititia* and *P. spinosa*) and cultivated species (*P. salicina* and *P. domestica*) and indicated that the molecular classification was not consistent with geographic origin or taxonomic status. Identification of intra and interspecific diversity is an important pre-requisite for promoting genetic diversity analysis. A larger sample throughout the distribution area of plums should be examined in order to detect and quantify the prevalent genetic diversity existing within and inter plum species at the molecular level. Wide genetic diversity is of great importance for developing improved varieties and estimating gene flow between cultivated species and wild-type species. Analyzing genetic diversity may be useful for designing effective breeding programs to broaden the genetic base of commercially grown varieties and plum improvement practices. To estimate the extent of variation in the plum germplasm between ecogeographical groups and related species, our results are useful for planning breeding programs involving diversity analysis, cultivar identification, or marker-assisted selection. The RAMPO database can be used for better management, conservation, resource utilization of plums.

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