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Full Length Research Paper

Phenotypic and nuclear DNA variation in Tunisian cultivars of date palm *(Phoenix dactylifera* L.)

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The aim of this study was to assess the morphological diversity of the five most important and widely consumed Tunisian date palm (*Phoenix dactylifera* L.) cultivars and the possible relationship between phenotypic variation and genome size and ploidy, since polyploidy can occur in this species. Five Tunisian palm date cultivars were evaluated, based on morphological traits and nuclear DNA content. The analysis of variance revealed statistically highly-significant differences among cultivars (Alig, Bsser, Deglet Nour, Kinticha and Hamra) for average palm length (LP) (m), perianth diameter (PD) (mm), pulp thickness (ThP) (cm), 30 fresh fruit weight (30 FrW) (g), 30 fresh seed weight (30 SW) (g), 30 SW/30 FrW ratio (%), fresh seed length (SL) (cm), fresh seed width (SWi) (cm) and water content (WaC) (%). The relationship among these characters was analyzed by principal component analysis (PCA), resulting in the separation of these cultivars into three groups. The first group included Alig and Deglet Nour, characterized by high values for perianth diameter, pulp thickness, 30 fresh fruit weight and water content. The cultivar Hamra (H) formed a separate group, characterized by high values for seed width, 30 seed weight, 30 seed weight /30fruit weight ratio and seed length. This study shows that certain Tunisian cultivars, apart from Deglet Nour (Alig and Hamra), are particularly recommended for future selection and breeding programs. The morphological variation was not due to differences in ploidy, since the tested cultivars had 2C nuclear DNA contents of 1.729 to 1.80 pg and were all diploids (2n = 2x = 36). The data suggested that the Tunisian cultivars have limited geographical distribution and that *P. dactylifera* is an ancestral species.

Key words: Date palm, fruit, genome size, morphological traits, seed.

INTRODUCTION

The date palm (*Phoenix dactylifera* L.) is a mono-cotyledon tree of the Arecaceae family and the Coryphoideae subfamily, widely cultivated for its fruits, the dates. Indeed, the date palm is grown in the arid and Saharan regions characterized by hot and dry summers, low and erratic rainfall and very low humidity during fruit ripening. The date palm is widespread in all warm areas of North Africa, the Sahara, from the Atlantic to the Red Sea, as well as in the Middle East to the Indus. Further north, it can be grown, but gives poor fruit. Dates are a major food and income source for local populations in the Middle East and North Africa and play significant roles in the economy in these regions. The highest export revenues are achieved by Tunisia, United States and Algeria. Tunisia is the world's largest exporter of date in value, about 100 million dollars per year (El Ferchichi and Hamza, 2008). But, in Tunisia, as in Algeria, new plantations have been carried out mainly over the last 30 years using a single cultivar, Deglet Nour. At the same time, the other, older cultivars are rarely propagated (Zehdi et al., 2004). This lack of interest for the cultivars of medium and low quality is an indirect cause of impoverishment of the genetic pool. The extension of the

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monovarietal culture can have harmful repercussions. Deglet Nour currently comprises 45% of the Algerian palm groves and approximately 60% of the Tunisian palm plantations, values which continue to grow (Rhouma, 2005). While other cultivars of lower quality are consumed in Tunisia (Kinticha, Alig, Bsser and Hamra), they are of less interest to the growers. Others constraints which restrict date palm culture and contribute to the decrease of genetic diversity of the palm groves in the Maghreb countries are drought, salinity, desertification, the old age of the palm trees and a vascular fusariosis caused by *Fusarium oxysporum* f. sp. *albedinis*, which remains the most serious disease (El Juhany, 2010). One consequence of all these biotic and abiotic stresses that the palm plantations undergo is the genetic erosion of the oases. It is necessary to safeguard this heritage through sustainable management.

Polyploidy is an important mechanism of diversification and generation of phenotypic variability in many flowering plants (Adams and Wendel, 2005). In addition, studies of molecular markers in this species are numerous (Sakka et al., 2004; Soliman et al., 2003), but polyploidy has been studied little in the date palm. As a model species for our study, we chose the date palm, *P. dactylifera* L. Thus, flow cytometry was used to estimate the nuclear DNA content of important Tunisian cultivars and hence, their ploidy level, together with a description of their morphological diversity in order to select discrimination criteria that can be used for future selection and breeding programs.

MATERIALS AND METHODS

Morphological study

Morphometric traits were determined for five varieties of *P. dactylifera*, Alig, Bsser, Deglet Nour, Kinticha, Hamra, originating from two sites, Tozeur (33°98'N, 8°22'E) and Kebili (33°8'N, 8°7'E), which contain the largest palm groves in Tunisia (Rhouma, 2005). Tunisian populations grow in Saharan climate conditions (Figure 1). For each variety in both sites we selected randomly five trees and made five measurements of each character per tree. Palms and fruits, taken randomly throughout the trees during September for Bsser and Hamra and in December for Alig, Kinticha and Deglet Nour, in 2009, were analyzed. The principal characteristics of the two sites studied are summarized in Figure 1. Trees were described by nine morphological characteristics: Average palm length (LP) (m), perianth diameter (PD) (mm), pulp thickness (ThP) (cm), 30 fresh fruit weight (30 FW) (g), 30 fresh seeds weight (30 SW) (g), 30 SW/30 FW ratio (%), fresh seed length (SL) (cm), fresh seed width (SWi) (cm) and water content (WaC) (%).

Nuclear DNA quantification

Plants were grown in a peat-soil mixture, in a controlled-environment chamber, for four weeks (14 h day, day/night temperature of 27/22°C and a photosynthetically-active radiation of 400 µmol m-2 s-1). For all cultivars, five plants were analysed for each variety and for each population. Estimation of nuclear DNA content was performed with a Partec PA II flow cytometer (Partec GMBH, Münster, Germany). Samples of growing leaf tissue of *P. dactylifera* and radish (*Raphanus sativus* L.) were prepared together. Radish has a 2C nuclear DNA content (1.11 pg; Doležel et al., 1992) similar to that of *P. dactylifera*. Leaf material was chopped with a razor blade for 60 s, in a 60 mm plastic Petri dish containing 0.4 ml of 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 µm filter into a 15 ml centrifuge tube. The Petri dish was washed twice with 0.8 ml of extraction buffer and the washings filtered into the 15 ml tube. After centrifugation at 1100 g for 10 min, the supernatant was removed and the pellet re-suspended in 1.6 ml of 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-1, respectively). All stages of the extraction were performed at 4°C. Samples were kept in the dark for 15 min at 37°C, before being filtered through a 30 µm 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 nuclear DNA content of *P. dactylifera* was estimated by the internal standard method, using the ratio of the *P. dactylifera*: radish G0/G1 peak positions (Doležel, 1997). The mean coefficient of variation (C.V.) (= (100 X standard deviation)/mean) ranged from 2.88 to 5.50%, depending on the variety. The equivalent number of base pairs was calculated assuming that 1 pg DNA = 978 Mbp (Doležel et al., 2003; Greilhuber et al., 2007).

Statistical analysis

A general linear model analysis of variance (ANOVA) was used to determine the effect of variety and site on all morphological traits. Differences between mean values were compared using the Duncan multiple range test (5%). Two-way ANOVA and separation of means were performed using XLSTAT 2010 software. Mean values of nuclear DNA were separated by the Student-Newman-Keuls test (5%), using SPSS v. 11.0.

Principal component analysis (PCA)

Principal component analysis (PCA) is a statistical technique used to replace the original variables with a number of basic dimensions, each of which is a linear combination of the original variables (Johnston, 1978; Mainley, 1994). So, in order to identify groups of inter-correlated variables for *P. dactylifera*, a PCA was carried out using the XLSTAT 2010 program on all individuals, for the five cultivars selected in the two nearby sites (Tozeur and Kebili).

RESULTS

Morphological study

For the five cultivars, the morphological traits analyzed showed C.V.s, ranging from 36.21% for the 30 SW/30 FrW ratio (%) to 7.20 and 7.40% for SL and SWi, respectively (Table 1). In spite of the observed intra-cultivar variation, the general linear model (ANOVA) revealed statistically-significant differences among the cultivars for all the examined characters ($P < 0.0001$). A significant difference was found between regions for the parameter LP (m) ($P < 0.02$), but not for PD (mm), ThP (cm), 30 FrW (g), 30 SW (g), 30 SW/30 FrW ratio (%),SL

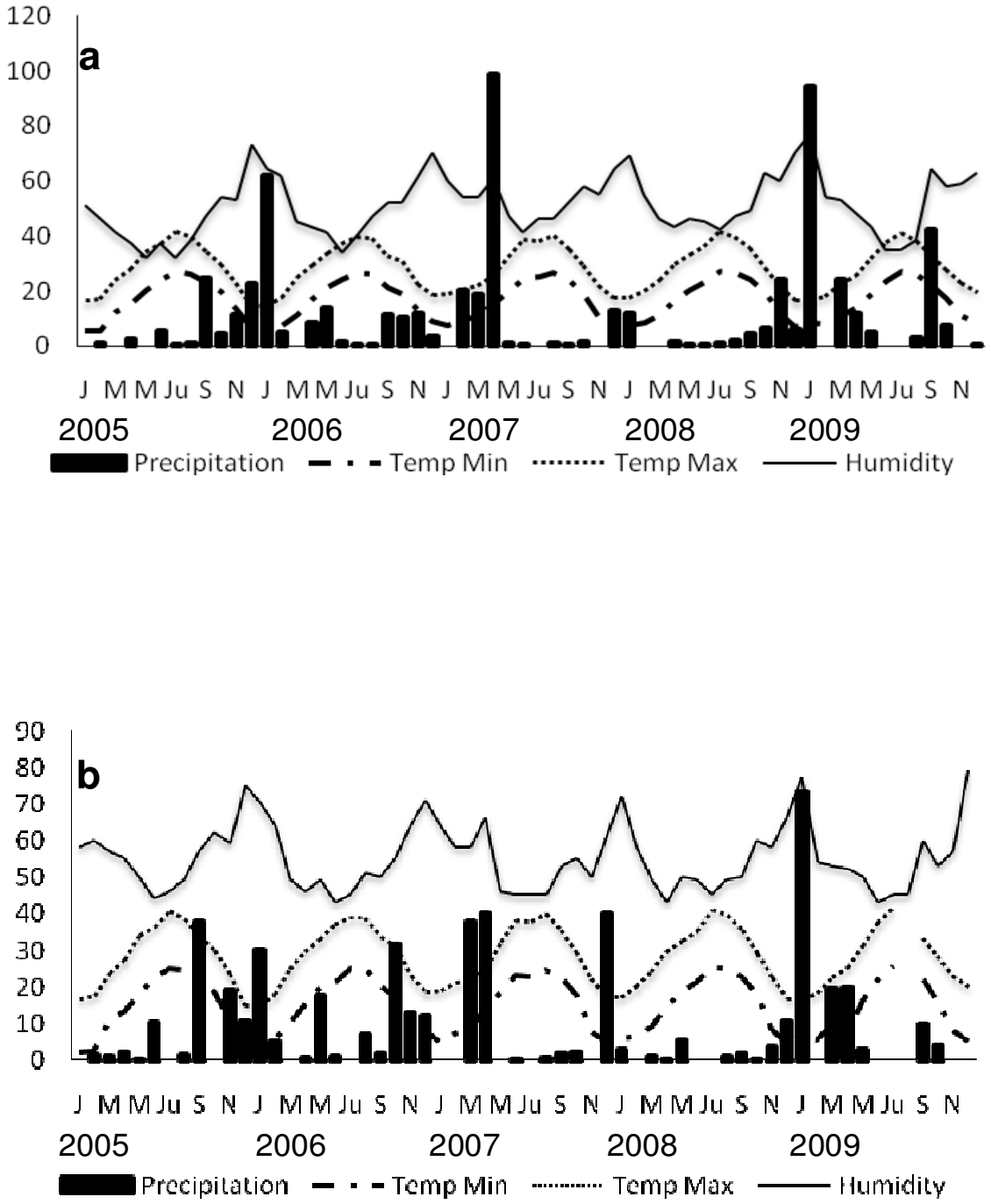

Figure 1. Rainfall, temperature and % humidity variation for five years, recorded at the stations of Tozeur (a) and Kébili (b). Precipitation (mm) is the monthly mean rainfall value. Temp Min (°C) is the monthly mean of the daily minima and Temp Max (°C) is the monthly mean of the daily maxima.

(cm), SWi (cm) or WC (%) ($P \geq 0.110$) (Table 1). The comparison of means (Table 2) revealed that the cultivar Deglet Nour had the highest values for LP (4.38 m), PD (10.58 mm), ThP (0.52 cm) and WC (30.36 %). Cultivar Alig showed the highest mean values for PD (10.68 mm), 30 FrW (295.27 g) and SL (2.52 cm), while cultivar Hamra showed the highest mean values for the 30 SW (52.22 g), 30 SW /30 FrW ratio (21.65%), SL (2.48 cm) and SWi (0.93 cm).

This study also revealed high positive correlation coefficients between palm length, perianth diameter and pulp thickness. The 30 fruit weight correlated positively with perianth diameter and pulp thickness. Water content correlated positively with pulp thickness. Seed width

Table 1. Descriptive statistics of characters measured in five cultivars, within two populations of *P. dactylifera* L. (mean character values, degree of freedom, standard deviation (SD), coefficients of variation (%) and the associated *F* and *P* values, for the significance of the differences).

Variable	Mean	Variety					Population				
		df	(SD)	C.V. %	F	P	df	(SD)	C.V. %	F	P
LP	3.72	4	0.45	12.09	3078.85	0.0001	1	0.45	12.09	5.1	0.02
PD	9.66	4	0.98	10.14	557.53	0.0001	1	0.98	10.14	1.4	0.23
ThP	0.41	4	0.08	19.51	439.47	0.0001	1	0.08	19.51	0.64	0.42
30 Fr W	230.5	4	54.9	23.80	45030.6	0.0001	1	54.9	23.80	0.0004	0.59
30 S W	31.34	4	9.64	30.75	17151.6	0.0001	1	9.64	30.75	0.2	0.69
30SW / 30FrW (%)	14.47	4	5.24	36.21	23422.4	0.0001	1	5.24	36.21	0.3	0.56
SL	2.36	4	0.17	7.20	148.24	0.0001	1	0.17	7.20	0.004	0.94
SWi	0.81	4	0.06	7.40	742.65	0.0001	1	0.06	7.40	0.3	0.57
WaC	22.46	4	5.22	23.24	408.99	0.0001		5.22	23.24	1.06	0.30

correlated positively with 30 seed weight and 30 seed weight/30 fruit weight ratio. High negative correlation coefficients were observed between palm length, 30 seed weight, 30 seed weight/30 fruit weight ratio and seed width. Pulp thickness correlated negatively with 30 SW/30 FW and SWi. Water content correlated negatively with 30 seed weight /30 fruit weight (Table 3).

Principal component analysis (PCA)

The first PC, which explained 60.63% of the total variability, is highly correlated with the 30 SW/30 FrW (%) ratio (+0.98), LP (+0. 88), ThP (+0.85), SWi (+0.65), 30 SW (+0.61), 30 FrW (+0.54) and PD (+0.52). The second PC explained 22.12% of total variance and is related to SL (+0.77). The third PC explains 9.44% of the total varia-tion and is correlated positively with the WaC (+0.57) (Figure 2). Furthermore, PCA revealed that agronomic traits related to 30SW/30FrW (%) ratio, LP, ThP, SWi, 30 FrW and PD accounted for a large proportion of the observed variability. According to Figure 3, the projections of five cultivars on the plane formed by the two components PCA1 and PCA2 show a first regrouping of cultivars (Alig (A) and Deglet Nour (N)) correlated with PCA1, characterized by the highest values for palm length, perianth diameter, thickness pulp, 30 fresh fruit weight and water content. In fact, within this group N and A were very close. The cultivars Bsser (B) and Kinticha (K) were placed in cluster II, correlated with the PCA2, with similar seed characteristics, while cultivar Hamra (H) formed a separate group (III), characterized by the highest values for seed width, 30 seed weight, 30 seed weight /30 fruit weight ratio and seed length, correlated with PCA1.

Flow cytometry

A histogram showing a typical flow cytometry analysis is shown in Figure 4. The C.V. of the *P. dactylifera* G0/G1peaks ranged from 2.88 to 5.50%.There were no significant differences in nuclear DNA content among the individuals of *P. dactylifera*. ($F = 0.588$, $P = 0.675$). The mean 2C nuclear DNA ranged from 1.73 to 1.80 pg (Table 4).

DISCUSSION

Morphological study

The quantitative character analyses of the five cultivars of *P. dactylifera*, from two nearby sites, revealed significant inter-cultivar differences for all the examined traits ($P \leq 0.0001$). However, the divergence between sites was relatively weak, reflecting their similar climatic conditions (Figure 1). The results showed significant difference between sites only for the average length of the palm (LP) ($P < 0.02$). The origin was not an important criterion for cultivar segregation, because interchange of cultivars is very frequent between oases (Saaidi, 1992). On the other hand, Elhoumaizi et al. (2002) noticed a great morphological variation between Moroccan date-palm cultivars, especially for leaf width, pinnae number, pinnae width at the middle, pinnae length at the bottom, spine length at the bottom and spine length and width at the top of the leaf. This is consistent with other studies that revealed the significance of the differences in vegetative characters among date palm cultivars, as in the study of Hussain et al. (1989) and Hamza et al. (2009). The importance of cultivars in each oasis is related to several factors, but most particularly to the quality parameters of their fruits: late maturity, firm texture, moisture tolerance, superior quality, long fruit stalk and sugar content; one of the most important fruit commercial characteristics. In a previous study, Elshibli (2009) found that sucrose and glucose contents of the fruits differed significantly among cultivars ($P < 0.0001$). Tunisian cultivars behave differently compared with all other groups, with sucrose

Table 2. Descriptive statistics (mean and standard deviation) for each morphological trait measured in 5 varieties within 2 populations of *P. dactylifera* L.

Variable	Tozeur population					Kébili population				
	Alig mean (SD)	Besser mean (SD)	Deglet Nour Mean (SD)	Kinticha mean (SD)	Hamra mean (SD)	ALig mean (SD)	Besser mean (SD)	Deglet Nour Mean (SD)	Kinticha mean (SD)	Hamra mean (SD)
LP	4.01 (0.11)b	3.29 (0.03)d	4.35 (0.02)a	3.76 (0.11)c	3.14 (0.02)e	4.04 (0.02)b	3.31 (0.03)d	4.38 (0.05)a	3.75 (0.02)c	3.17 (0.01)e
PD	10.62 (0.17)a	8.03 (0.37)c	10.56 (0.2)a	9.55 (0.11)b	9.44 (0.58)b	10.68 (0.27)a	8.11 (0.38)c	10.58 (0.28)a	9.57 (0.26)b	9.54 (0.22)b
ThP	0.48 (0.04)b	0.39 (0.02)c	0.50 (0.01)a	0.35 (0.03)d	0.32 (0.02)e	0.47 (0.02)b	0.38 (0.03)c	0.52 (0.01)a	0.34 (0.01)d	0.31 (0.03)e
30 Fr W	294.58 (1.72)a	170.39 (1.06)d	288.48 (3.79)b	168.13 (0.92)e	230.96 (2.4)c	295.27 (2. 13)a	170.42 (0.8)d	287.62 (2.57)b	167.35 (1.35)e	231.12 (1.81)c
30 S W	26.02 (1.24)d	29.33 (0.38)b	23.40 (0.36)e	27.81 (0.25)c	50.22 (0.88)a	26.04 (0.25)d	29.24 (0.29)b	23.21 (0.32)e	27.79 (0.17)c	50.06 (0.66)a
30 S W/ 30 Fr W (%)	8.82 (0.41)d	17.21 (0.25)b	8.1 (0.15)e	16.54 (0.19)c	21.74 (0.52)a	8.81 (0.10)d	17.15 (0.16)b	8.06 (0.15)e	16.60 (0.16)c	21.65 (0.26)a
SL	2.50 (0.01)a	2.13 (0.2)d	2.38 (0.14)b	2.32 (0.01)c	2.48 (0.01)a	2.52 (0.01)a	2.06 (0.12)d	2.40 (0.05)b	2.35 (0.01)c	2.46 (0.01)a
SWi	0.75 (0.01)e	0.8 (0.02)c	0.77 (0.01)d	0.82 (0.01)b	0.93 (0.01)a	0.75 (0.01)e	0.79 (0.01)c	0.78 (0.02)d	0.81 (0.02)b	0.92 (0.01)a
WaC	24.45 (0.02)b	23.82 (0.11)b	30.36 (0.26)a	16.03 (0.07)d	18.23 (0.01)c	24.45 (0.01)b	23.84 (0.02)b	29.14 (0.6)a	16 (0.08)d	18.21 (0.02)c

Means followed by different letters (a to e) are significantly different according to the Duncan test ($P < 0.05$).

content as high as 63.0% and glucose content as low as 6.3%. The results of our study show that the fruit and seed properties differed in their ability to differentiate among cultivars; for example, Deglet Nour can be differentiated according to its average palm length, pulp thickness and water content, while 30 fruit weight and seed length significantly differentiated the cultivar "Hamra". Elshibli and Korpelainen (2009) reported significant differences ($P < 0.001$) for Sudanese cultivars in relation to fruit weight, flesh weight and fruit and seed sizes, which expressed a wide range of diversity among cultivars. Furthermore, this morphological study of Tunisian palm date cultivars reveals that some characters were closely correlated with each other. Palm length was positively correlated to perianth diameter and pulp thickness. Seed length was strongly correlated to 30 fruit weight and perianth diameter. If the seed was long, the fruit weight was high and therefore, the female flower developed an important perianth diameter.

We can distinguish some clusters of our cultivars according to their fruit characteristics. In fact, the PCA results show grouping of cultivars based on fruit and seed traits. So we can distinguish a cluster composed by Deglet Nour and Alig, which have high values of palm length, perianth diameter, thickness pulp, 30 fruit weight and water content. In addition, we can discern two other cultivar clusters according to their seed length (cluster II), high seed width, high 30 seed weight, high 30 seed weight /30 fruit weight ratio and high seed length (cluster III). Furthermore, Principal component analysis (PCA) revealed that agronomic traits related to 30SW/30FrW (%) ratio, LP, ThP, SWi, SWi, 30 FrW and PD accounted for a large proportion of the observed variability. In addition, these results can be taken into account if we consider that certain cultivars as Alig (A), Bsser (B) or Kinticha (K) are not less important than Deglet Nour and can be adopted for the

Table 3. Correlation coefficients between morphological characters for each cultivar.

Parameter	LP	PD	TP	30 Fr W	30 SW	30 S W/30 Fr W (%)	SL	SWi	WC
LP									
PD	0.79**								
Th P	0.80**	0.53**							
30 FrW	0.58**	0.78**	0.66**						
30 SW	0.82**	0.29**	0.73**	-0.17*					
30 SW/ 30FrW (%)	0.92**	0.68**	0.91**	0.74**	0.79**				
SL	0.25**	0.69**	0.14*	0.62**	0.18**	-0.26*			
S Wi	0.75**	0.31**	0.76**	0.31**	0.91**	0.81**	n.s		
Wa C	0.38**	0.19**	0.58**	0.43**	0.39**	-0.53**	n.s	0.43**	

ns, Non-significant; * significant (P < 0.05); ** highly significant (P ≤ 0.01). LP, Average palm length; PD, perianth diameter; TP, pulp thickness; 30 FrW, 30 fresh fruit weight; 30 SW, 30 fresh seed weight; SL, fresh seed length; SWi, fresh seed width; WC, water content.

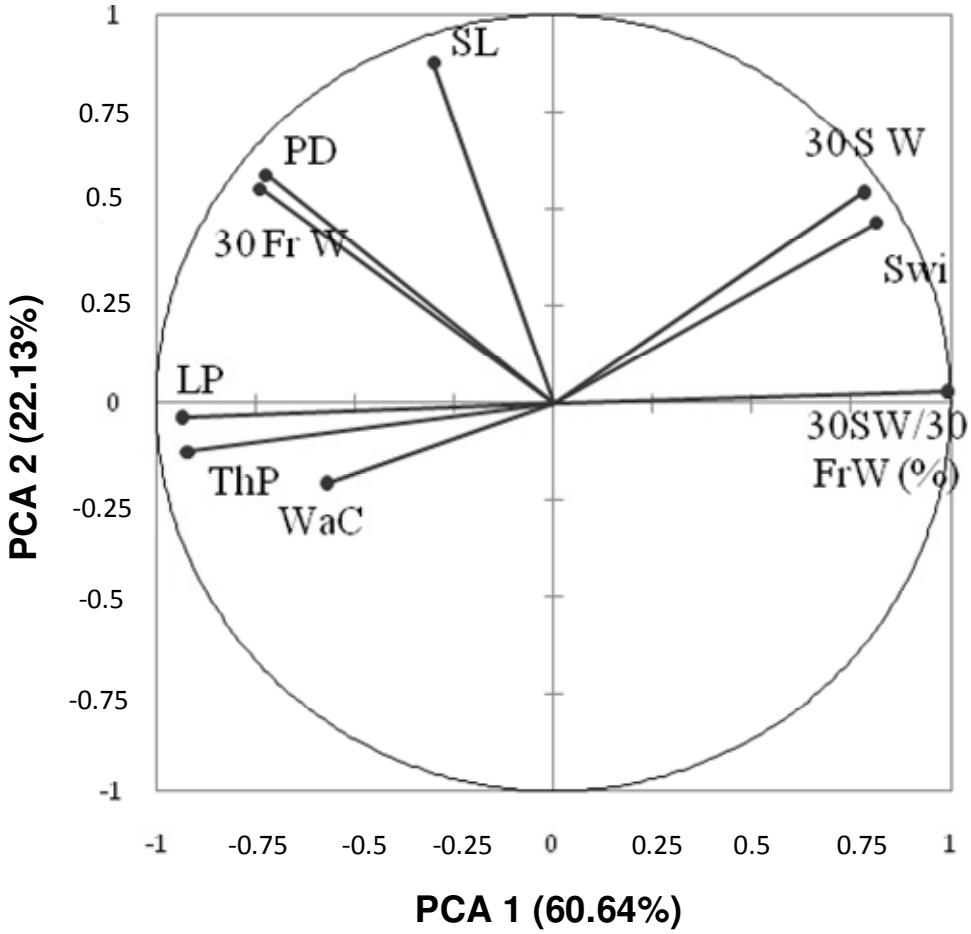

Figure 2. Representation of date-palm variables on the plane 1 to 2 of the principal component analysis. Codes indicate variables: (LP) length of palm, (PD) perianth diameter, (ThP) thickness pulp, (30 FrW) 30 fruits weight, (30 SW) 30 seeds weight, (30 SW/30 FrW) 30 seeds weight/30 fruits weight ratio, (SL) seed length, (SWi) seed width and (WaC) water content.

renewal of some palm groves, since it is necessary to avoid the exaggerated and sometimes sole use of Deglet Nour for the renewal of oases.

The data obtained in this study show the great variability of the date palm fruits and seeds collected from the two main Tunisian oases. These results underline the

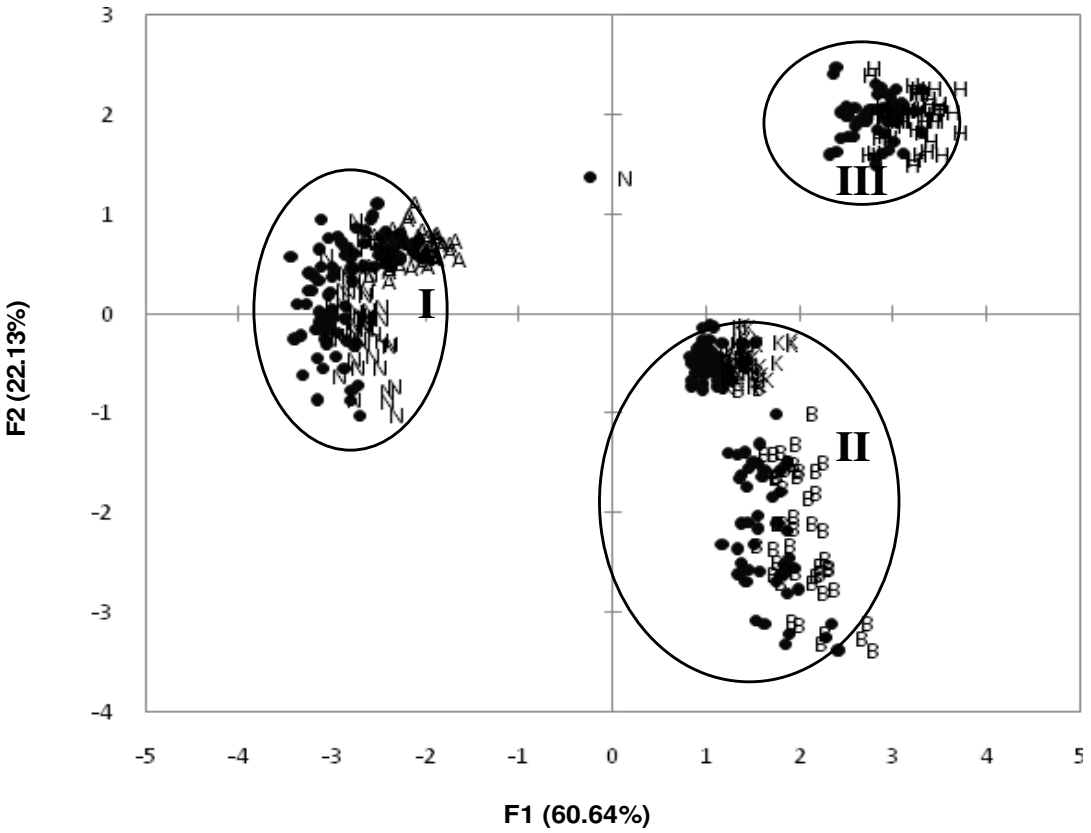

Figure 3. Plot of the cultivars on the first and second components. Codes indicate cultivars: (A) Alig, (N) Deglet Nour, (B) Bsser, (K) Kinticha and (H) Hamra.

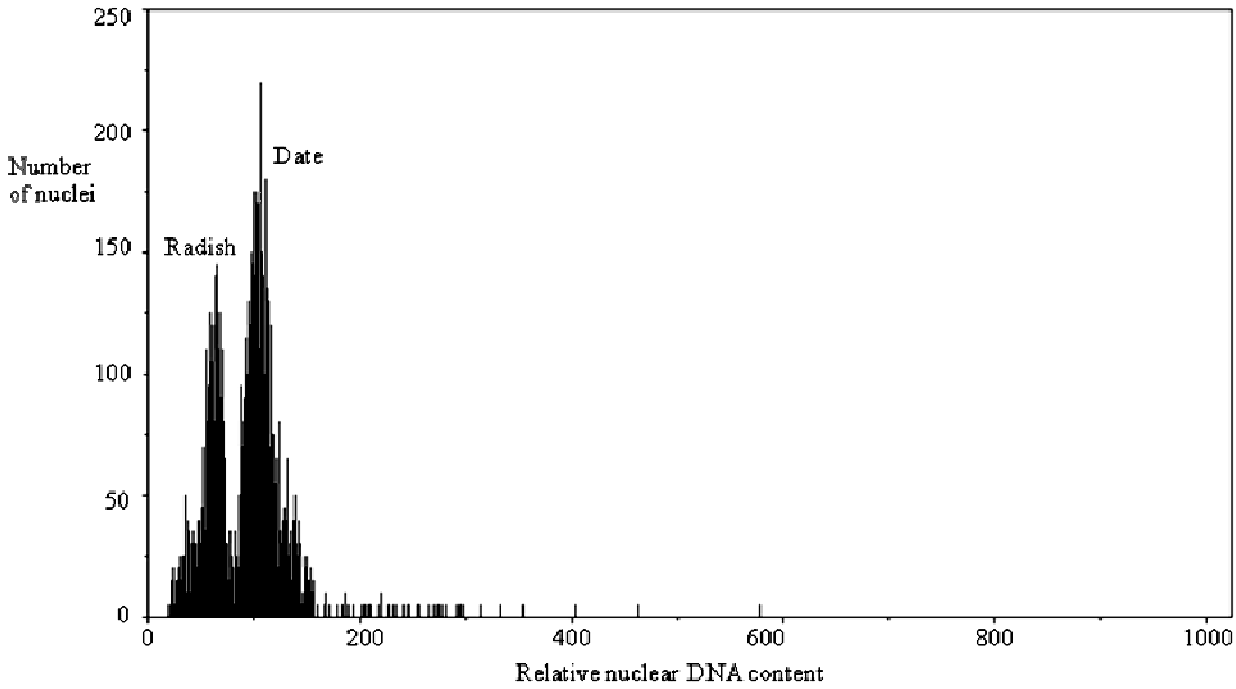

Figure 4. Flow cytometric analysis of date (*P. dactylifera* L.*)* variety Hamra. Leaf cell nuclei were stained with propidium iodide, using radish (*R. sativus* L.) as internal standard (2C nuclear DNA content = 1.1 pg).

Table 4. Mean nuclear DNA (2C) amount ± SD (n = 5) and genome size for the studied varieties of *P. dactylifera* L., determined by flow cytometry.

Variety	Mean 2C nuclear DNA amount (pg) ± SD	1C genome size (Mbp)
Alig	1.764 ± 0.057	863
Bsser	1.773 ± 0.091	867
Deglet Nour	1.729 ± 0.075	845
Kinticha	1.781 ± 0.098	871
Hamra	1.801 ± 0.052	881

importance of preserving the genetic resources of date palm and could be used in clonal selection. Moreover, the development of cultivars more adapted to current and future demands of marketing must take into consideration a strategy that prevents genetic erosion; otherwise, the reduction of the biodiversity of this species is a real risk. Thus, evaluation of the biodiversity in date palm trees is a fundamental step for the implementation of a conservation strategy.

Genome size and ploidy level

Flow cytometry offers a simple, rapid and accurate method for determining nuclear DNA content and ploidy levels in plants, but has not been applied extensively in date palm (Srisawat et al., 2005). Our results show that genome size did not vary significantly in the studied Tunisian cultivars of *P. dactylifera*. All were diploid, since the mean 2C nuclear DNA was between 1.73 and 1.80 pg. These results suggest a low intraspecific variation, at least among the studied cultivars and no polyploidy or aneuploidy - unlike the information reported by Al-Ani et al. (2010). So, differences in ploidy did not contribute to the observed morphological variation. This reflects the situation for *Atriplex halimus* L. and *Acacia tortilis* (Forsk.) Hayne ssp. *raddiana* (Savi) Brenan species, for which only tetraploid individuals were detected within Tunisia and for *Ceratonia siliqua* L., for which only diploid plants were found (El Ferchichi Ouarda et al., 2006, 2008, 2009). It seems that this taxon presents ancient and stable characters; According to Ortúñez and de la Fuente (2004), there is correlation between ploidy level and geographical distribution: diploid species with small genome size have a restricted distribution, whereas species of greater genome size show a wider distribution area. Leitch et al. (1998) analysed angiosperm C-values in a phylogenetic context and concluded that ancestral taxa probably possess small genomes with 1C ≤ 3.5 pg. Previous studies have looked at biochemical and molecular markers of date palm cultivars. The use of isozymes permits discrimination of some cultivars, but these methods gave contradictory results when applied to date-palm trees and other plants (Chevreau, 1990; Elhoumaizi et al., 1993). Research in molecular markers is taking the lead in this context (Aitchitt et al., 1995; Corniquel and Mercier, 1997; Lewis et al., 2000).

However, these methods are at a preliminary stage with regard to date-palm research; they have been tested only on a limited number of cultivars (Benabdellah et al., 2000; Trifi et al., 2000). Despite the large number of cultivars evaluated by Sedra et al. (1998) (43 cultivars using RAPD markers), the study could not identify a criterion that discriminated significantly among cultivars. A wide range of variability has been reported on date palm; however, no links have been identified among genetic variability and the observed morphological and/or physiological traits (Saker et al., 2000; Azeqour et al., 2002; El-Assar et al., 2005; Saker et al., 2006).

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