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MICROSATELLITE DIVERSITY AMONG TUNISIAN DATE PALM (*PHOENIX DACTYLIFERA* L.) SUBPOPULATIONS

HAMZA HAMMADI[1], GIOVANNI GIUSEPPI VENDRAMIN[2] AND FERCHICHI ALI[1]

[1]Arid and Oases Cropping Laboratory, Arid Area Institute Medenine, 4119, Tunisia
[2]Plant Genetics Institute, National Research Council, 50019 Sesto Fiorentino, Florence, Italy

Abstract

Phoenix dactylifera L. is native to the southern of Tunisia where it is the most important crop. The aim of this study is to assess the diversity in Tunisian date palm cultivars using molecular markers. The use of reliable and stable vegetative features on 26 cultivars showed clusters characterized also by fruit traits such as consistency and maturity period. Microsatellites analysis supports this statement and it was carried out by using markers with high polymorphism. Analysis of molecular variance revealed significant genetic variation among fruit subpopulations ($p < 0.05$). Semi soft fruit subpopulation has significant differentiation with soft and semi dry fruit subpopulations. These results suggest that continental Tunisian date palm cultivars are not a unique population which is in opposition with a unique one ancestral date-palm population and this result is the first to be published in *P. dactylifera*.

Introduction

Date palm (*Phoenix dactylifera* L.) is one of the most important fruit crop in the world. It is extensively grown as a food crop and covers about 3% of the cultivated area of the world (Dowson, 1982). In Tunisia, the domestication process of this species has been started since some thousand years. Actually, more than 4 millions date palm trees are cultivated in Tunisian oases (Rhouma, 2005) offering a wide socio-economic stability.

Morphologically, *Phoenix dactylifera* L., is a perennial dioecious monocotyledon species (2n=36). This dioecious nature causes a high genetic diversity (Munier, 1981) which is widely observed in Tunisian date palms. In this area many reports (Rhouma, 1994, 2005; Ferchichi & Hamza 2008) noted that there are more than 200 cultivars. Among these cultivars, a high genetic variation has been noticed especially in reproductive characters. Hence, cultivar identification is usually based on its fruit characteristics. Despite most of the morphological traits are under environmental factors, they have been used for the characterization of date palm cultivars in Tunisia (Ben, Salah 1993; Rhouma, 1994, 2005; Ben Salah & Hellali, 2004), California (Nixon, 1950), Morocco (Elhoumaizi *et al.*, 2002). Morphological features are reliable when they are stable under different growth conditions and differ among subpopulations or cultivars. Hamza *et al*., (2009) proposed six vegetative morphological traits which can be used in several condition oases. These characters have a strong genetic control when the environment component is discarded. The results showed significant relationship between vegetative traits and fruit ones such as consistency and maturity period. Tunisian continental date palm can be divided into several subpopulations according to their fruit characteristics with vegetative indicators. The correlation between vegetative and fruit traits may be genetically and/or the result of an adaptation with the local condition (Smouse, Rutgers University, 'pers. comm.').

Date palm genetic diversity was also evaluated by fruit analytic parameters (Booij *et al*., 1992; Reynes *et al*., 1994; Bouabidi *et al*., 1996) and isoenzymes markers (Baaziz & Saaidi, 1988; Ould Mohamed Salem *et al*., 2001) but they have a low degree of polymorphism and they are usually affected by environment and plant physiology (Al-Jibouri & Adam, 1990; Lawe *et al*., 2004; Rhouma 2008). Azeqour *et al.,* (2002) demonstrated that some isoenzyme markers have a high variation and correlated with two morphological characters, presence of inflorescences and offshoots formation. Indeed, the development of molecular tools has revealed high levels of DNA polymorphism in the genome. Many DNA markers have been applied for detecting molecular variation and for searching relations with diseases essentially the Bayoud one (Louvet & Toutain, 1973). Several markers have been used such as random amplified polymorphic DNA (RAPDs) (Sedra *et al*., 1998; Trifi *et al*., 2000; Al-Khalifa & Askari, 2003), inter simple sequence repeats (ISSRs) (Zehdi *et al*., 2002), random amplified microsatellite polymorphism (RAMPO) (Rhouma, 2008) and Amplified fragment length polymorphisms (AFLP) (Rhouma, 2007). These markers revealed usually a high polymorphism among date palm cultivars but it remains difficult to characterize cultivars. Codominant markers like simple sequence repeats (SSRs) are very useful to identify date palm cultivars. Such markers have detected a high polymorphism in Tunisian date palm populations (Zehdi *et al.*, 2004). Most of these molecular work showed that the geographic origin didn't affect the grouping of Tunisian cultivars which is in agreement with the unique Mesopotamian domestication origin of this crop (Wrigley, 1995).

This study focuses on the *Phoenix datylifera* L., using molecular markers to elucidate relationship date palm subpopulations morphologically distinct (Hamza *et al.*, 2009). Microsatellite loci developed by Billote *et al.,* (2004) were used to determine the genetic relationships between these groups and characterize their levels of diversity.

Material and Methods

a. Date palm genotypes: The date palm materials were collected from many localities of the Tunisian continental oases. These areas represent more than 85% of the total date palm oases of Tunisia. Twenty-six cultivars were chosen for their good fruit quality and are the most common genotypes in the main plantations located in the south of Tunisia (Ferchichi & Hamza, 2008). Table 1 summarizes some characteristics of the studied cultivars.

b. DNA preparation: Genomic DNA of each genotype was extracted from young leaves. Total nuclear DNA was extracted according to Invisorb® Spin Plant Mini Kit (Invitek). DNA polymorphism was detected by polymerase chain reaction (PCR) using SSR markers being developed for *Phoenix dactylifera* L., by Billotte *et al.,* (2004) (Table 2). Thus, five markers were used to study the genetic relationships of individuals and subpopulations, chosen for their highly expected heterozygosis values (Billotte *et al.*, 2004).

c. PCR amplification: It was performed according to Markus (2000) with simple modification in a volume of 12.5 µl contained 50 ng of genomic DNA**,** 5X Green GoTaq® reaction buffer (Promega), 0.2 mM of dNTPs, 0.625 U of Taq polymerase (Go*Taq*, Promega), 2 mM of $MgCl_2$, 0.2 µM of each primer. Amplifications were carried out in DNA amplification Thermocycler (GeneAmp® PCR System 9700). The

conditions for SSR–PCR were an initial denaturation 94°C for 3 min followed by 10 cycles of denaturation at 94°C for 20 s, annealing at primer specific melting temperature for 1min, extension at 72°C for 40 s, followed by 25 cycles of 30 s at 94°C, 30 s at 53°C and 30 s at 72°C with a final extension at 72°C for 8 min. The amplification products were detected using electrophoresis with 1% agarose gels and by staining with Ethidium bromide. For final analyses, 0.54 µl of amplified DNA and 5 µl of MagaBACE ET400-R DNA Size Standard were loaded. Genotyping was carried out using an automatic DNA analyser, MegaBACE 1000.

d. Data analysis: All molecular data were computed with Genalex program (Version 6) (Peakall & Smouse, 2006). The genetic diversity was estimated by the determination of the total number of alleles and genotypes per locus. The observed and expected heterozygosity (H_o and H_{exp}) (Nei, 1987) and the inbreeding coefficients *F*is were also assessed. Genetic distances (PhiPT) between groups were tested by Analysis of Molecular Variance (AMOVA) (Excoffier *et al.*, 1992) and bootstrapped (999 times). In addition, the individual microsatellite genotypes scores were coordinated in a bi-dimensional space by principal component analysis (PCA) by computing the genetic distance matrix. Hierarchical classification was conducted by calculating Nei *et al*'s., Da genetic distance (1983) using Populations 1.2.28 Software (Langella, 2002). The distance matrix obtained was used to construct the dendrogram using the Unweighted pair group-method (UPGMA). Bootstrap values were computed over 2000 replications.

Results

a. Genetic diversity analysis: The SSR profiles exhibited 36 alleles with an average of 7.2 alleles per locus. The microsatellite markers were found to be highly polymorphic with the number of alleles ranging from six to eight among the 26 cultivars genotypes (Table 2).

High levels of expected (H_{exp}) and observed (H_o) heterozygosity were observed, the mean H_{exp} value for all loci is 0.63 and the H_o values ranged from 0.34 (mPdCIR093) to 0.88 (mPdCIR010) indicating that the Tunisian date palms collection is characterised by a high degree of genetic diversity. The observed heterozygosity was less than the expected one within population heterozygosity for mPdCIR032, mPdCIR070 and mPdCIR093 loci, as shown by the positive *Fis* values, 0.04, 0.33 and 0.47 respectively (Table 2). This indicates an overall excess of homozygosity within populations compared with that expected under random mating. However, the deviation from Hardy Weinberg equilibrium was significant only for mpdCIR010 ($p<0.01$) and for mpdCIR093 ($p<0.001$) (Table 2).

b. Subpopulations differentiation: The application of vegetative morphometric data showed a subpopulation differentiation based on fruit characteristics. However, it is necessary to verify this dissimilarity for the studied date palms genotypes so that morphological and molecular variation patterns could be directly compared. In the scattergram, a subpopulation separation can be observed. Concerning the maturity period, earlier cultivars are also associated and they are opposed to later maturity cultivars (Fig. 1). For the fruit consistency, the semi soft cultivars are easily grouped (Fig. 2). The consistency subpopulations dendrogram discern two clusters: the soft and semi dry cultivars group and the second with dry and semi soft cultivars, for the maturity period subpopulations, two groups are distinguished, the first with season and late maturity cultivars and the second with earlier ones (Fig. 2).

Table 1. Name, origin and main characteristics of the studied date-palm genotypes.

No.	Name	Oases	Fruit characteristics		
			Color	Consistency	Maturity
1.	Alig	Nefzaoua, Jerid	Dark brown	Semi-dry	Late
2.	Ammary	Nefzaoua, Jerid	Black	Soft	Early
3.	Bejjou	Nefzaoua, Jerid	Brown	Dry	Late
4.	Bissr Helou	Nefzaoua, Jerid	Pale brown	Dry	Season
5.	Choddakh	Nefzaoua, Jerid	Dark Amber	Semi-Soft	Season
6.	Choddakh Ben Jbir	Nefzaoua	Dark Amber	Semi-soft	Season
7.	Deglet Nour	Nefzaoua, Jerid	Amber	Semi-soft	Late
8.	Dhahbi	Jerid	Amber	Semi-soft	Late
9.	Fezzani	Nefzaoua, Jerid	Amber	Semi-dry	Season
10.	Fermla	Nefzaoua	Brown	Semi-dry	Season
11.	Ghars souf	Nefzaoua, Jerid	Dark brown	Soft	Season
12.	Gondi	Nefzaoua, Jerid	Amber	Semi-soft	Season
13.	Gosbi	Nefzaoua, Jerid	Black	Soft	Early
14.	Hamra	Nefzaoua, Jerid	Amber	Semi-dry	Season
15.	Hissa	Nefzaoua, Jerid	Honey	Soft	Early
16.	Hlwa	Nefzaoua	Honey	Semi-dry	Late
17.	Horra	Nefzaoua, Jerid	Amber	Dry	Season
18.	Kintichi	Jerid	Reddish	Dry	Late
19.	Loghrabi	Jerid	Dark brown	Semi-soft	Season
20.	Om Leghlez	Jerid	Amber	Soft	Early
21.	Rtotbayet elmansoura	Nefzaoua	Brouwn	Soft	Season
22.	Rotbayet yagouta	Nefzaoua	Dark amber	Soft	Early
23.	Rtob Houdh	Nefzaoua, Jerid	Amber	Soft	Season
24.	Tezerzayet Kahla	Nefzaoua, Jerid	Black	Soft	Season
25.	Tezerzayet Safra	Jerid	Dark brown	Soft	Early
26.	Tronja	Nefzaoua, Jerid	Dark brown	Semi-dry	Late

Table 2. Genetic diversity indices for five microsatellites loci revealed in the studied Tunisian date palm genotypes. (*A*, observed number of alleles per locus; G, Observed number of genotypes per locus; H_o, observed heterozygosity; H_{exp}, expected heterozygosity; *Fis*, Fixation index) (**p<0.01, ***p<0.001)

Locus	Allelic range (bp)	A	G	H_{exp}	H_o	Fis
mPdCIR010	141-181	8	9	0.75	0.88	-0.17**
mPdCIR015	141-157	6	12	0.70	0.80	-0.14
mPdCIR032	305-321	7	11	0.72	0.69	0.04
mPdCIR070	206-228	8	12	0.69	0.46	0.33
mPdCIR093	178-197	7	11	0.65	0.34	0.47***

The AMOVA analysis (Table 3) on several fruit consistency subpopulations found out that 93% of the variation was significantly attributed to the variation within subpopulations ($p<0.05$). The pairwise comparisons (Table 3) showed significant genetic differences between the fruit consistency subpopulations. The semi soft date palm subpopulations were found to be significantly different from the most other examined subpopulations. Significant genetic differences were not observed among maturity period subpopulations.

Table 3. AMOVA of date palm microsatellite variations. Pairwise population PhipT values and percentage of molecular variance (999 permutations) across five microsatellite loci for date palm subpopulations.

	Pop 1	Pop 2	PhipT values	P	Percentage of molecular variance		
					Among	Within	P
Fruit consistency subpopulations	Soft	Semi soft	0.107	0.025*	7%	93%	0.031*
	Soft	Semi dry	0.004	0.391			
	Soft	Dry	0.050	0.193			
	Semi soft	Semi dry	0.164	0.027*			
	Semi soft	Dry	0.060	0.239			
	Semi dry	Dry	0.021	0.374			
Maturity period subpopulations	Early	Saison	0.009	0.372	0%	100%	0.542
	Early	Tardive	0.034	0.258			
	Saison	Tardive	0.000	0.420			

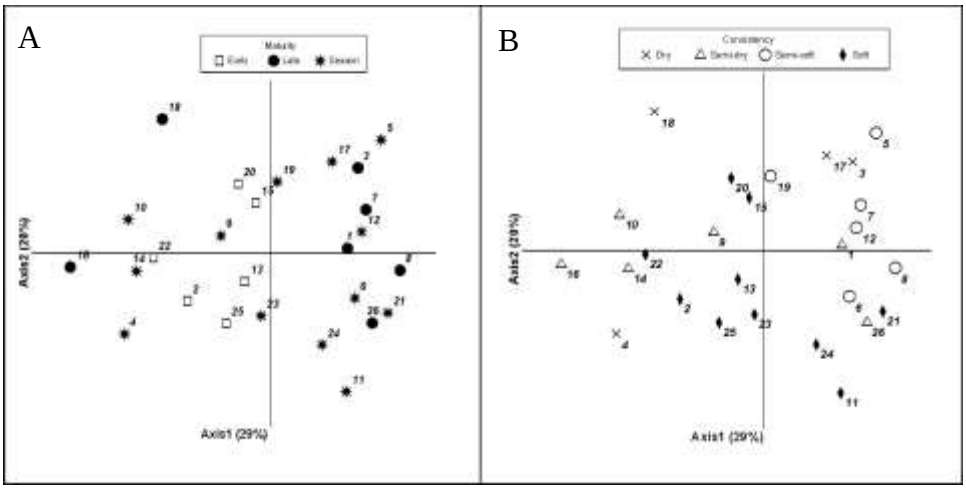

Fig. 1. Scattergram showing relative position of 26 date palm cultivars defined by the first two principal components based on the genetic distance of five microsatellite loci.

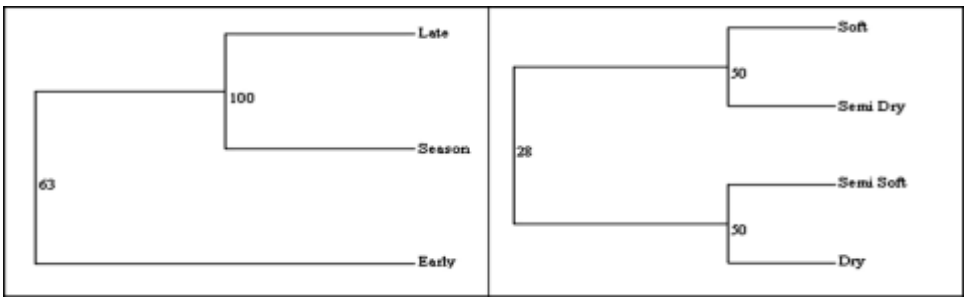

Fig. 2. Unweighted pair group-method (UPGMA) dendrogram of the consistency and maturity period subpopulations based on Nei *et al*., Da (1983) genetic distance. Bootstrap values are given in percentage over 2000 replications.

Discussion

The task of resolving the genetic relationships of date palm cultivars using vegetative morphometric data is very difficult because of the high phenotypic plasticity of the genus (Munier, 1973; Sedra *et al.*, 1993, 1996). According to our previous studies (Hamza *et al.*, 2009), we have selected some vegetative traits that have not significant adaptive response to the environment. Important results are shown when we have practised these characters on several date palm genotypes from different continental oases. This study showed that the selected vegetative traits are useful for fruit characters identification. Interested correlation between vegetative traits and fruits ones are underscored. In the present work, we performed SSR genotyping method in order to examine the genetic diversity in Tunisian date palms. Data exhibited evidence of the utility of this technology to enlarge the number of markers suitable for evidencing molecular polymorphisms in this crop. As a result, a large number of SSR alleles have been revealed with a mean of 7.2 per locus and permitted to detect a relatively high degree of genetic variability in this crop. In fact, the scored values of diversity are higher at the intra group level than at the inter groups level. Similar results have been reported in Moroccan, Algerian and Tunisian date palm cultivars using isozyme markers (Bennaceur *et al.*, 1991; Fakir, 1992; Ould Mohamed Salem *et al.*, 2001). These results can be attributed to the dioecious nature of this crop.

No location effect was noted for growth rate, indicating that none of the genotypes shows a marked preference for one site over the other. This agrees with Trifi *et al.*, (2000) and with Zehdi *et al.*, (2002, 2004), who suggest that groupings of Tunisian date palm are made independently from their geographic origin. This argued for the existence of one Tunisian date palm population rather than separate regional populations. These works also suggest a common genetic basis, which is in agreement with a unique one Mesopotamian domestication origin of this crop (Wrigley, 1995). Elshibli & Korpelainen (2008) have shown that Morocco date palm cultivars are significantly different compared to Sudan ones because of the geographic distance reflecting the difficulties in exchanging plant materials. In our study, although the cultivars are almost from the same oases, we found a population's differentiation. Analysis of molecular variance (AMOVA) revealed that most of the genetic variability detected was contained within fruit consistency subpopulations (93%). Estimates of inter-subpopulations genetic variation, calculated from PhiPT, were high in the case of Semi Soft group compared with Semi Dry and Soft ones and these values are very low substantially when maturity period subpopulations were compared.

Tunisian date palms are usually considered as a unique population (Trifi *et al.*, 2000; Zehdi *et al.*, 2004) because it's relatively too small to create a geographic distinction during the cultivation period. The distinction of semi soft cultivars reflects that Tunisian date palm can be divided, at least, into two subpopulations with different origin. Their introduction in Tunisian oases was under anthropogenic pressure that's why we found the four consistency groups in the same oasis discarding the geographic effect.

The correlation of fruit consistency and genetic structure date palm subpopulations is intriguing. This evidence suggests that the Tunisian date palms may not be a unique population and this result is the first to be published in *P. dactylifera*. The genetic distinctness of the Semi soft subpopulation supports this statement. However, given the small number of populations and the examined loci this interpretation should be viewed as preliminary. Additional cultivars and loci will also allow for a wider range of population genetic analysis to be conducted.

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