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Full Length Research Paper

Analysis of Tunisian date palm germplasm using simple sequence repeat primers

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Inter Simple Sequence Repeat (ISSR) markers involve polymerase chain reaction (PCR) amplification of DNA using a single primer composed of a microsatellite sequence. ISSR technology rapidly reveals high polymorphic fingerprints and determines genetic diversity. We used genetic markers generated from selected ISSR primers to assess genetic diversity among a set of Tunisian date palm varieties. Seven primers were used to cluster 12 date palm varieties and 77 polymorphic markers were sufficient to identify all of the varieties. These discrete molecular markers were used to estimate genetic distances among the 12 accessions and to examine their genetic relationships. Data analysis identified phenetic groups that were in agreement with those obtained according to agronomic traits and random amplified polymorphic DNA (RAPD) markers. Among the 12 simple sequence repeat (SSR) motifs tested, the most abundant were AG.

Key words: Genetic diversity, ISSR polymorphisms, date palm, *Pheonix dactylifera*.

INTRODUCTION

In Tunisia as in several tropical countries, oasis cultures consist of date palm groves. These are the major factors of oases environmental and economic stability. Its utilization consists of a large number of adapted ecotypes. This long-lived dioecious is of a great socio-economic interest. Firstly, date palm is the main factor of oases farming and the under-covered crops. Secondly, it constitutes the principal financial resources of oasiens since dates are used either for food or many other commercial purposes. For instance, more than 10% of Tunisian population depend on date palm's culture where more 250 cultivars have been inspected (Rhouma, 1994). However, Tunisian plantations are characterized by the prevalence of the elite variety called "Deglet Nour" in spite of their large genetic diversity. This trend would contribute significantly to the genetic erosion in this important phytogenetic patrimony and accelerate its vulnerability to biotic and abiotic stresses. Most North African date palm plantations have been seriously threatened for several decades, such as the brittle leaves disease that is of unknown cause and the vascular fusariosis due to the fungus *Fusarium oxysporum* f. sp. *albedinis*. (Djerbi et al., 1985; Haddouch, 1996). In Tunisia, this important tropical crop is currently in danger by severe genetic erosion. Hence, it is imperative to elaborate a strategy aiming at the evaluation of the genetic diversity and the preservation of the Tunisian date palm germplasm. In this scope, many reports using either morphological traits or isozyme makers to identify the Tunisian date palm varieties have been published (Rhouma, 1994; Reynes et al., 1994; Ould Mohamed Salem et al., 2001). Moreover, data based on molecular markers such as RFLPs and RAPDs have been used to characterize date palm genotypes (Sedra et al., 1998, Ben Abdallah et., al 2000, Trifi et al., 2000, Trifi, 2001).

Thus it has been assumed that the identified markers are of some suitability in the date palm varieties identification. Therefore, the search of many other markers is required to obtain a deeper comprehension of

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the genetic organization in Tunisian date palm varieties. It is noticeable that among the markers that can be investigated, inter Simple Sequence Repeat (ISSR) is one of the mos eftficient. This strategy is of several benefits over the other procedures (isozymes, RFLPs and RAPDs). First, it permits detection of polymorphisms in microsatellites and intermicrosatellites loci without previous knowledge of the DNA sequence. Microsatellites regions are abundant throughout the eukaryotic genome, are highly polymorphic in lengh (Tautz, 1989) and are interspersed (Gupta et al., 1994, Zietkiewicz et al., 1994). Secondly, ISSR is informative about many loci and are suitable to discriminate closely related genotype variants (Fang and Roose, 1997). And lastly, ISSR markers constitute discrete markers suitable in the DNA fingerprinting (Gupta et al., 2000)

In the aim to improve date palm culture, we become interested in the use of the microsatellites as sustainable molecular markers to examine the polymorphisms in a Tunisian date palm collection. The ISSR strategy was therefore performed to access the DNA diversity among crop genotypes. Similar strategy has been made to distinguish ecotypes in closely related groups such as fruit crops, orange, citrus and vigna (Fang et al., 1997; Fang and Roose, 1999; Stepansky et al., 1999; Ajibade et al., 2000). Here we report the employment of ISSRs as informative markers to investigate the examination of the phylogenic relationships among a set of Tunisian date palm varieties.

Table 1. Tunisian date palm varieties used in this study. (*) nomenclature according to Rhouma (1994); (*), (**) and (***) varieties also called Alligue, Menakher and Rochdi, respectively.

Variety name	Label	Oasis	Origin
Deglet Nour	1	Tozeur	Tunisia
Boufagous	2	Djérid	Tunisia
Ftimi *	3	Djérid	Tunisia
Kenta	4	Djerid	Tunisia
Kintichi	5	Djérid	Tunisia
Deglet Bey **	6	Degache	Tunisia
Ghars Mettig	7	Degache	Algeria
Zehdi	8	Tozeur	Iraq
Arichti ***	9	Djérid	Tunisia
Khou Ftimi	10	Djérid	Tunisia
Horra	11	Djérid	Tunisia
Okht Deglet	12	Degache	Tunisia

MATERIALS AND METHODS

Plant material

A set of 12 date palm varieties that are listed in Table 1 was investigated. These were chosen for their good fruit quality and are the most common genotypes in the main Tunisian plantation. Among these varieties, two that are recently introduced (one from Iraq and the other from Algeria) were included in the study. The plant material consists of young leaves provided from the Centre de Recherches Phoénicicoles, INRAT, Degache, Tunisia. Date palm trees (one for each genotype) were randomly chosen and sampled directly from the oases in the South of Tunisia.

DNA preparation

Total DNA was extracted from frozen young leaves of adult trees according to Dellaporta et al. (1984) protocol with little modifications. After purification, DNA concentrations were determined using a Gene Quant spectrometer and its integrity was checked with agarose minigel electrophoresis according to Sambrook et al. (1989).

Primers and ISSR assay

A total of 12 primers were tested to amplify the isolated DNA. These primers listed in Table 2, and their composition has been arbitrarily established.

For PCR amplifications, a 25 µl reaction mixture was used and it contained between 20 and 30 ng of total cellular DNA (1 µl), 60 pg of primer (1 µl), 2.5 µl of 10X Taq DNA polymerase reaction buffer, 1.5 unit of Taq DNA polymerase (Quantum-Appligène, France) and 200 mM of each dNTP (DNA polymerisation mix, Amersham-Pharmacia, France). Each reaction mixture was overlaid with 25 µl of mineral oil to avoid evaporation during PCR cycling. Amplifications were performed in DNA amplification Thermocycler (Crocodile III, Quantum-Appligène, France). The apparatus is programmed to execute the following conditions: a denaturation step of 5 min at 94°C, followed by 35 cycles composed of 30 s at 94°C, 90 s at the annealing temperature, and 90 s at 72°C. A final extension of 72°C for 5 min was included.

To reduce the possibility of cross contamination in the amplification reactions, a master reaction mixture is routinely prepared and a control was used. This control consists of the reaction mixture excluding any DNA matrix. Amplifications were performed at least twice and only reproducible products were taken into account for further data analysis.

Amplification products were separated on 1.4% agarose gels in 0.5X TBE buffer and detected by staining with ethidium bromide (0.5 µg ml^{-1}) according to Sambrook et al. (1989).

Data analysis

For Each DNA sample, ISSR bands were transformed into a binary matrix where the presence of reproducible polymorphic DNA band at particularly position on gels is scored 1, while a 0 denotes its absence. The matrix was computed with the Gendist program (version 3.572c) (PHYLIP package), using the formula of Nei and Li (1979) to generate the genetic distance matrix. The distance matrix was then employed to draw the precise relationships between the date palm varieties. The resultant treefiles were then submitted to the TreeView (Win32; 1.5.2) software to map a dengrogam. All this analyses were carried out using PHYLIP (phylogeny inference package, version 3.5c) (Felsenstein, 1993).

RESULTS

A total of 12 primers were screened for their ability to generate consistently amplified band patterns and to access polymorphism in the tested varieties. Among

Table 2. List of ISSR primers used in this study.

Sequence	Annealing Temperature	Amplified bands		
		Total	Polymorphic bands	% Polymorphism
(TGGA) 5	55°C	0		
(GACA) 4	45°C	0		
(ACTG) 4	45°C	6	0	0%
(GACAC) 4	55°C	10	0	0%
(AG)10	55°C	Smear		
(AGG) 6	55°C	13	13	100%
(AG)10 G	60°C	17	16	94%
(AG)10 C	60°C	14	13	93%
(AG)10 T	57°C	14	14	100%
(CT)10 A	57°C	7	7	100%
(CT)10 G	60°C	10	9	90%
(CT)10 T	57°C	12	10	83%

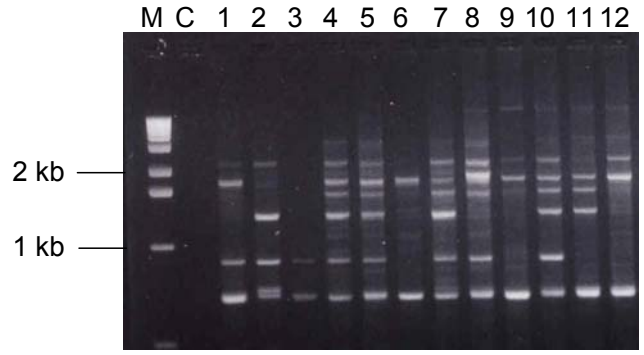

Figure 1. Example of ISSR polymorphism banding patterns in a subset of Tunisian date palm varierties using (AG)10C primer. M: Standard molecular size marker (1 Kb Ladder, Gibco-BRL); Lane labelled C: control included; lanes (1-12): accessions described in materials and methods and Table 1. Sizes of molecular weight markers are indicated in kb.

these primers, only 9 revealed polymorphic and unambiguously scorable bands. While smear or no amplified products were observed with the other primers.

These 9 primers generated 7 to 16 polymorphic DNA bands with an average of 9.11 bands with a range of 200 to 2500 bp. Typical amplified products are reported in Figure 1. The polymorphic patterns obtained suggested that the ISSR procedure constitutes an alternative approach that is suitable to examine the date palm's genetic diversity at the DNA level. A total of 77 polymorphic ISSR products was obtained.

The matrix has a genetic distance of 0.3008 to 0.7885, with a mean of 0.505. Thus, it may be assumed that the varieties are characterized by a high degree of genetic diversity at the DNA level. The smallest distance value of 0.3008 was observed between Zehdi and Ghars Mettig varieties indicating that these ecotypes are the most similar. The maximum distance value (0.7885) suggesting high divergence was detected between Khouftimi and Boufagous varieties. The phenogram obtained (Figure 2) informs supports the varietal clustering.

The cluster are similar to those based on agronomic traits particularly related to the fruits. This is well exemplified in the case of Boufagous and Deglet Bey that are characterized by dates of a large size and of a dark colour. Note that in this tree branching, the foreigner varieties (ie Zehdi and Ghars Mettig) are unlikely clustered with the indigenous ones. Hence our results agree with the Mesopotamian origin of date palm domestication (Wrigley, 1995).

DISCUSSION

In this study we have designed the ISSR technology in order to enlarge the number of molecular markers that are suitable in the characterization of a Tunisian date palm collection. Our data provide evidence of a genetic diversity between the tested varieties indicating the interrelationship between the date palm ecotypes in spite of their agronomic divergence. Currently, date palms cultivars' selection by farmers is particularly based on date fruity and locally adapted genotypes. Consequently, only a small part of date palm genome that concerns mainly genes encoding these agronomic traits is affected by this selective way and suggests a narrow genetic diversity among the selected genotypes. On the whole, our data augment those describing the application of molecular tools in date palm variability analysis and previously reported (Sedra et al., 1998; Trifi et al., 2000).

We observed the foreign date palm varieties used in this study (the Algerian Ghars mettig and Iraqi Zehdi) did not significantly diverge from the autochthonous ones, concurring with the ancient date palm's Mesopotamian

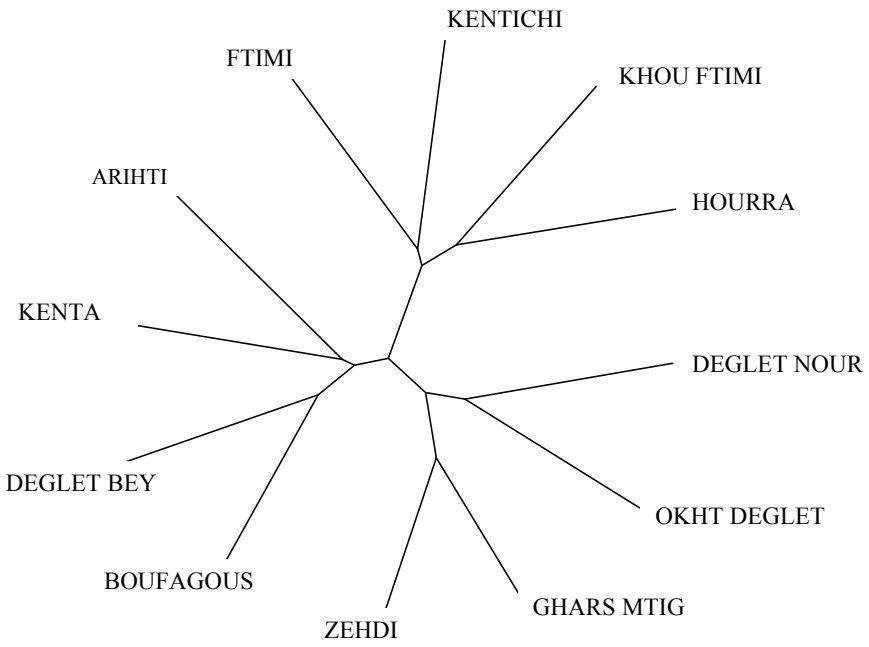

Figure 2. Dendrogam of 12 Tunisian date palm varieties constructed from Nei and Li's formula genetic distance matrix estimated from ISSR data and clustred with the UPGMA.

(fertile crescent) domestication origin (Munier, 1974; Wrigley, 1995). On the other hand, dendrogram showed that accessions grouping in relation to their geographical origin are not well defined. Consequently, since all date palm ecotypes are originated by hybridization, it may be assumed that they have a common genetic basis. Nevertheless, varieties diverged from others by mutational events that arise during selection. Obviously, in the present study we have not explored the molecular basis of divergence in ISSR markers (mutation events that concern the length of simple sequence repeats and the insertion/deletion in the sequence).

A large-scale characterization of ISSR would allow a precise fingerprinting of date palm germplasm grown world-wide and would provide molecular markers that can discrimination between cultivars.

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