International Journal of Botany

ISSN: 1811-9700

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International Journal of Botany 6 (2): 182-186, 2010
ISSN 1811-9700

Genetic Diversity of Tunisian Date Palm Germplasm Using ISSR Markers

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Abstract: Aim of the study is the analysis of the genetic diversity among a set of Tunisian date palm varieties. We used different genetic markers generated from selected ISSR primers. 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. Seven primers were used to cluster 10 date palm varieties and 82 polymorphic markers were sufficient to identify all of the studied varieties. These discrete molecular markers were used to estimate genetic distances among the 10 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. Present result provides evidence of divergence between Tunisian varieties that are organized in clusters. However, we are unable to identify all tested genotypes as mono varietal groups.

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

INTRODUCTION

In Tunisia as in several Sub-tropical countries, oasis cultures consist of date palm groves as main cash crop. 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. However, Tunisian plantations are characterized as more than 250 cultivars (Rhouma, 1994). Prevalence of the elite variety called Deglet Nour, in spite of their large genetic diversity, it's been widely cultivated. This trend of cultivation 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 causal and the vascular fusariosis due to the fungus *Fusarium oxysporum* f. sp. *albedinis* (Djerbi, 1988). In Tunisia, this important 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 aiming at 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; 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 could have suitability in the date palm varieties identification. Therefore, the search of many other. markers is required to obtain a deeper comprehension of 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 most efficient (Gupta and Varshney, 2000). 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 (Gupta *et al*., 1994). Microsatellites regions are abundant throughout the eukaryotic genome,

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are highly polymorphic in lengh (Tautz, 1989) and are interspersed (Zeitkeinicz *et al*., 1994). Secondly, ISSR is informative about many loci and are suitable to discriminate closely related genotype variants (Roose and Fang, 1997). And lastly, ISSR markers constitute discrete markers suitable in the DNA fingerprinting.

Microsatellites as sustainable molecular markers to examine the polymorphisms in a Tunisian date palm collection was used. 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, i.e., orange, citrus and vigna (Fang *et al*., 1997; Fang and Roose, 1999; Stepansky *et al*., 1999; Ajibade *et al*., 2000).

Here we report the use of ISSRs as informative markers to investigate the examination of the phylogenic relationships among a set of Tunisian date palm varieties.

MATERIALS AND METHODS

Plant material: A set of 10 date palm varieties 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 those 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 en Agriculture Oasienne (CRRAO), Degache, Tunisia. Date palm trees (one for each genotype) were randomly chosen and sampled directly from the oases in the South of Tunisia (Table 1). Newly developed leaves were sampled from adult trees labelled and taken to the Laboratory for analysis. This study is completed within the framework of the research tasks of the laboratory and which lasted 6 months since December 2008 at June 2009.

DNA preparation: Total DNA was extracted from frozen young leaves of adult trees according to Dellaporta *et al.* (1985) protocol with little modifications (Teixeira da Silva, 2005). After purification, DNA concentrations were determined using a Gene Quant spectrometer and its integrity. Both were checked on agarose minigel 0.8% electrophoresis according to Sambrook *et al.* (1989).

Primers and ISSR assay: A total of 12 primers (Invitrogen/illumnia company) were tested to amplify the isolated DNA, only 7 primers were shown reproductibl and polymorphic band, these primers and their composition were listed in Table 2.

Table 1: Tunisian date palm varieties used in this study. Nomenclature according to Rhouma (1994)

Variety name	Label	Oasis	Origin
Deglet nour	1	Tozeur	Tunisia
Mnker	2	Djerid	Tunisia
Berhi	3	Djerid	A.Saoudit
Kenta	4	Djerid	Tunisia
Kintichi	5	Djerid	Tunisia
Boufaggous	6	Degache	Tunisia
Ghars mettig	7	Degache	Algeria
Zehdi	8	Tozeur	Iraq
Goundi	9	Djerid	Tunisia
Khou ftimi	10	Djerid	Tunisia

Table 2: List of ISSR primers used in this study

	Amplified bands			
Sequence	Annealing temperature	Total	Polymorphic bands	Polymorphism (%)
(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
(ACTG) 4	45°C	7	0	0
(GACAC) 4	55°C	9	0	0
(TGGA) 5	55°C	2	0	0
(GACA) 4	45°C	3	0	0
(AG) 10	55°C	5	0	0

For PCR amplifications, a 25 μL reaction mixture was used and it contained between 20 and 30 ng of total cellular DNA (2 μL), 60 pg of primer (1 μL), 2.5 μL of 10X Taq DNA polymerase reaction buffer, 1.5 unit of Taq DNA polymerase (Promega, France) and 200 mM of each dNTP (DNA polymerisation mix, Promega, France). Amplifications were performed in DNA amplification Thermocycler (Biorad, icycler). The apparatus is programmed as following profile: a denaturation step of 4 min at 94°C, followed by 40 cycles composed of 30 sec at 94°C, 45 sec at the annealing temperature and 180 sec 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 2% agarose gels in 1X TAE buffer and detected by staining with ethidium bromide (0.5 mg 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 particularity 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 tree files were then submitted to the TreeView (Win32; 1_5.2, R.D.M). software to map a dendrogram. All this analyses were carried out using PHYLIP (phylogeny inference package, version 3.5c, Department of Genetics, University of Washington, Seattle, Washington, USA Felsenstein).

RESULTS

A total of 12 primers were used for their ability to generate consistently amplified band patterns and to access polymorphism in the tested varieties for biodiversity. Among these primers, only 7 revealed polymorphic and unambiguously scorable bands. While smear or no amplified products were observed with the other primers.

These 7 primers generated 7 to 16 polymorphic DNA bands with an average of 11.71 bands with a range of 200 to 2500 bp; typical amplified products are reported in Fig. 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 82 polymorphic ISSR products were 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 maximum distance value (0.7885) suggesting high divergence was detected between Khouftimi and Boufagous varieties. The phenogram obtained informs supports the varietal clustering. The cluster in Fig. 2 is similar to those based on agronomic traits particularly related to the fruits, 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 one, concurring with the ancient date palm's Mesopotamian (fertile crescent) domestication origin (Munier, 1974; Wrigley, 1995). This is well exemplified in the case of Boufagous and Menaker that are characterized by dates of a large size and of a dark color. Note that in this tree branching, the foreigner varieties (i.e., Zehdi and Ghars Mettig) are unlikely clustered with the indigenous ones.

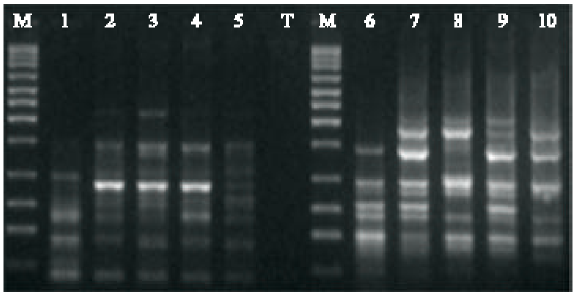

Fig. 1: Example of ISSR polymorphism banding patterns in a subset of Tunisian date palm varieties using (CT)10G primer. M: Standard molecular size marker (1 Kb Ladder, Proméga); Lane labelled T: control included; lanes (1-10): accessions described in materials and methods and Table 1. Sizes of molecular weight markers are indicated in kb

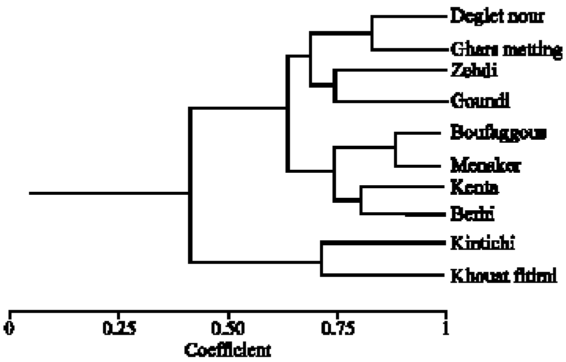

Fig. 2: Dendrogam of 10 Tunisian date palm varieties constructed from Nei and Lie's formula genetic distance matrix estimated from ISSR data and clustered with the UPGMA

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). Dendrogram (Fig. 2) 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 divergened from others by mutational events that arise during selection. The smallest distance value of 0.3008 was observed between of Boufagous and Menaker varieties indicating that these ecotypes are the most similar.

Obviously, in the present study we have not explored the molecular basis of divergence using 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 discriminate between cultivars.

These patterns permit only the analysis of the DNA divergence and the phylogenetic construction among Tunisian date-palm varieties. On the whole, the varieties' relationships based on the molecular data concur in some cases with the groupings based on the high degree of the variation observed for several agronomic traits particularly related with the fruits produced by each variety. This is well pointed out in the case of Boufegous and Menaker varieties clustered together with the smallest distance value of 0.3008 indicating that these ecotypes are the most similar, which produce dates currently characterized by a relatively big size and similar flavor and color qualities. On the other hand the organization deduced from the ISSR markers' analysis is in agreement with previous molecular studies (Sedra *et al.*, 1998). Sedra *et al.* (1998) these authors showed that morphologically similar varieties are associated to the fruit quality and it was observed that cultivars originated from Algeria and Iraq did not show a far relation from the Tunisian ecotypes suggesting a narrow genetic diversity of date-palms between them relatively low polymorphism and the lack of evidence on organization observed among the date palm varieties grown in Tunisia could be related to the mode of introduction and way of germplasm maintenance. Foundation, of germplasm, is somewhat limited. The fact that the cultivars from Algeria and Iraq did not markedly differ from the genetic diversity present in varieties from Tunisia suggesting that a narrow genetic diversity between populations from those varieties, have been derived and maintained over several centuries, was shown. Moreover, sexual reproduction and seedling selection may also have played a role (Oliur Rahman, 2007).

CONCLUSIONS

Aim of the study was to provide polymorphic DNA markers for examination of the phylogenetic relationship among different Tunisian date-palm varieties using ISSR technology. As a result of our investigation we may expect that the subsequent banding patern computed using appropriate programs, would be useful for the establishment of phylogenetic relationships among a set of high quality fruit varieties. Our data provides evidence of divergence between Tunisian varieties that are organized in clusters. However we are unable to identify all tested genotypes as mono-varietal groups. In combination with morphological criteria, ISSR assay and fingerprinting could allow the establishment of a catalogue of cultivars grown world-wide for, identification of duplicate accessions and establishment of a core collection.

Apparently present study provides evidence that ISSR method is an alternative strategy to precisely assess the phylogenetic relationships between a set of Tunisian date-palm varieties. It is obviously necessary to enlarge both the number of varieties and/or the number of primers to obtain a deeper insight of Tunisian date-palm varieties. A search is currently in progress to molecularly characterize these varieties in shorter step and molecular markers those can identify disease-tolerance as well.

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