MOLECULAR CHARACTERIZATION OF TUNISIAN DATE-PALM GERMPLASM USING ISSR MARKERS

Zehdi S.1, Trifi M.1, Ould Mohamed Salem A.1, Rhouma A1,2 and Marrakchi M.1

1: Laboratoire de Génétique, Immunologie & Biotechnologie, Faculté des Sciences de Tunis, Campus Universitaire, 2092 El Manar Tunis, Tunisia (North Africa)

2: Centre de Recherches Phoénicicoles, INRAT, Degache, Tunisia

ABSTRACT

In Tunisian, as in several tropical countries, oasis cultures consists of date-palm groves, are major factors of environmental and economic stability. However, most North African date-palm plantations have been seriously threatened for several decades, by a vascular fusariosis due to the Fungus *Fusarium oxysporum* f. sp. *albedinis.* Until now, Tunisian plantations appear to have been spared. However, they are continuously menaced by this fusariosis due to its rapid propagation.

In order to establish a preventive strategy for Tunisian date-palm groves, many studies have been developed aiming at the characterization of date-palm varieties. In our work, ISSR strategy is used to molecularly characterize ecotypes. This study has provided a large number of potential polymorphic markers suitable in the establishment of the phylogenetic relationships among Tunisian cultivars.

Data analysis identified phyletic groups composed of varieties clustered together, however, they do not constitute monophyletic groups.

Key words: PCR, ISSR polymorphisms, phylogenic relationships, Tunisian date-palms

INTRODUCTION

In Tunisia as in several tropical countries, oasis cultures consist of date-palm groves. These are the major factors of oasis environmental and economic stability. Its utilization consists of a large number of adapted ecotypes locally called cultivars. However, most North African date-palm plantations have been seriously threatened for several decades, either by abiotic or biotic stresses causing diverse plagues 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 deterioration due to the predominance of the elite Deglet Nour variety in Tunisian plantations. In spite of their increased phytogenetic resources, (Rhouma 1994). 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 have been designed and described the use either morphological traits or isozyme makers to identify the Tunisian date-palm varieties (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 developed to molecularly 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 and in the examination of their phylogenetic relationships. 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, microsatellites are the more efficient ones: first, microsatellites are interspersed in the genomes (Gupta *et al* 1994, Sanchez *et al* 1996); second they constitute discrete markers suitable in the DNA fingerprinting and third, microsatellites are informative about many loci and are suitable to discriminate closely related genotype variants (Fang & Roose 1997).

Aim into improve date-palm culture, we became interested in the use of the microsatellites as sustainable molecular markers to examine the polymorphisms in a Tunisian collection. The inter simple sequence repeat (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 & 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. As a result, using appropriate primers a large number of DNA stretches were amplified and were adjusted to estimate DNA diversity and relatedness in date-palms.

MATERIALS AND METHODS

Plant material

A set of 12 varieties, listed in table 1 has been used. These varieties were chosen for their good fruit quality and being the most common genotypes in the main Tunisian plantation. Among these varieties, two that are recently introduced (one from Iraq Deglet Nour and one from Algeria, Ghars Mettig) were included in the study. The plant material consists of young leaves provided from the «Centre de Recherches Phoenicicoles, 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 cellular DNA was Extracted from frozen young leaves of adult trees according to Dellaporta's *et al.* (1984) with minor modifications. After purification, DNA concentrations were determined using a Gene Quant spectrometer and its integrity was proved by agarose minigel electrophoresis according to Sambrook *et al* (1989).

Primers and ISSR assay

A total of 12 random primers were tested to amplify DNA using total cellular DNAs as matrix.

For PCR amplifications, a 25 µl reaction mixture was used, containing between 20 and 30 ng total cellular DNA (1 µl), 60 pg primer (1 µl), 2.5 µl Taq DNA polymerase reaction buffer, 1.5 unit Taq DNA polymerase (Quantum-Appligene, France) and 200 mM 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 amplification is program was as follows: a denaturation step of 5 min at 94°C, followed by 35 cycles, each is composed of 30 seconds at 94°C, 90 seconds at the primer specific melting temperature ™, and 90 seconds at 72°C, and a final extension of 72°C for 5 min.

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 0.5 × TBE buffer and detected by staining with ethidium bromide (0.5 µg ml-1) according to Sambrook *et al* (1989).

Data analysis

The PCR generated band profiles were compiled into a binary data matrix. Data were then computed with the Gendist program (version 3.572c) to produce a genetic distance matrix using the formula of Nei & Li (1979). The genetic distance matrix was then computed using the UPGMA cluster analysis. Felsenstein's appropriate programs in PHYLIP (phylogeny inference package, version 3.5c) were used to carry out all these analyses (Felsenstein 1993).

RESULTS

A total of 12 primers were screened for their ability to generate consistently amplified banding patterns and to access polymorphism in the tested varieties. Among these primers, only 9 have revealed polymorphic and unambiguously scorable bands, while smear or no amplified products generated by the other ones.

On the other hand, using the primers that are characterized by their ability to generate multiple banding profiles, 7 to 16 polymorphic DNA bands with an average of 9-11 bands per oligonucleotide in a ranging size of 200 - 2500 bp were currently amplified. Typical amplified banding profiles are reported in figure 1. In addition, expectedly one oligonucleotide amplified no polymorphic DNA banding profile, while all the other ones have revealed polymorphic patterns, suggesting that the ISSR procedure constitutes an alternative approach that is suitable to examine the date-palm's genetic diversity at the DNA level. This is strongly supported especially with the large number of polymorphic ISSR products (a total of 77) which is higher than in other cultivated crop spices such as wheat (Kojima *et al* 1998) and grapvine (Moreno *et al* 1998).

The implied varieties distance genetic matrix (Table 3) indicates an range from 0.3008 to 0.7885 with a mean of 0,505. Thus, it may be assumed that the implied 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 Khou Ftimi and Boufagous varieties.

The use of UPGMA algorithm permitted to cluster the data and to draw the relationships between the tested accessions. The ensuing phenogram reported in fig. 2 indicates the genetic divergence described above and supports the varietal clustering.

Hence, it is assumed that all the implied accessions are clustered together. The identified groups supported significantly three main divergent clusters. The first one is composed of Ftimi, Kintichi, Kou Ftimi et Hourra varieties. The second cluster is composed of Deglet Nour, Okht Deglet, Ghars Mtig, Zehdi, Boufagous, Deglet Bey, Kenta et Arichti varieties. The resultant cluster groupings corresponded 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 foreign varieties (ie Zehdi and Ghars Mettig) are unlikely to cluster 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 molecular characterization and the phylogenic relationships to examine in a Tunisian date-palm collection. Our data provide evidence of a genetic diversity between the tested accessions. Thus, it may be ensued that all date-palm ecotypes are interrelated in spite of their agronomic divergence. This consideration is strongly supported if regarding the cultivars' selection mode in date-palms applied by farmers that is particularly based on date quality 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. Over all our data agree with those describing the application of molecular tools in date-palm variability analysis and previously reported (Sedra *et al* 1998; Trifi *et al* 2000). The present work also provides evidence that the ISSRs appear effective to explore the molecular polymorphism and the phylogenic relationships in date-palms. It is clearly evident that in combination with agronomic parameters, isoenzyme and RAPD markers, ISSRs could provide the establishment of identification criteria in date-palm germplasm.

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

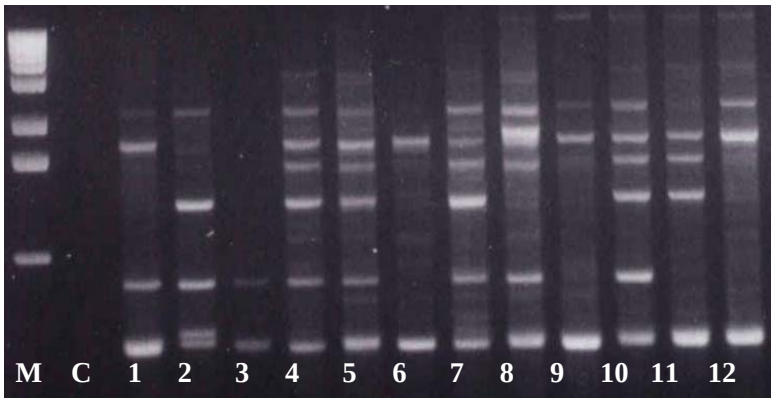
Variety name1	Label	Oasis	Origin
Deglet Nour	1	Tozeur	Tunisia
Boufagous	2	Djérid	Tunisia
Ftimi *	3	Djérid	Tunisia
Kenta	4	Djerid	Tunisia
Kintichi	5	Djérid	Tunisia
Deglet Bey **	6	Degache	Tunisia
Ghars Mettig	7	Degache	Algeria
Arichti ***	8	Djérid	Tunisia
Khou Ftimi	9	Djérid	Tunisia
Horra	10	Djérid	Tunisia
Okht Deglet	11	Degache	Tunisia
Zehdi	12	Tozeur	Iraq

Table II: Characteristics of the tested primers and the amplified bands

N°	Sequence	Amplified bands		
		Total	Polymorphic	%
1	(AGG) 6	13	13	100%
2	(TGGA) 5	**	**	
3	(ACTG) 4	0	0	0%
4	(GACA) 4	0	**	**
5	(GACAC) 4	10	0	0%
6	(AG)10	Smear	**	**
7	(AG)10 G	17	16	94%
8	(AG)10 C	14	13	93%
9	(AG)10 T	14	14	100%
10	(CT)10 A	7	7	100%
11	(CT)10 G	10	9	90%
12	(CT)10 T	12	10	83%

Figure 1 : Typical examples of ISSR amplified banding profiles using Tunisian date-palm varieties. Primer tested (AG)10C (Panel A) and (CT)10G (Panel B). M : Standard molecular weight size, C : control, 1-12 : sampled varieties

Panel A



Panel B

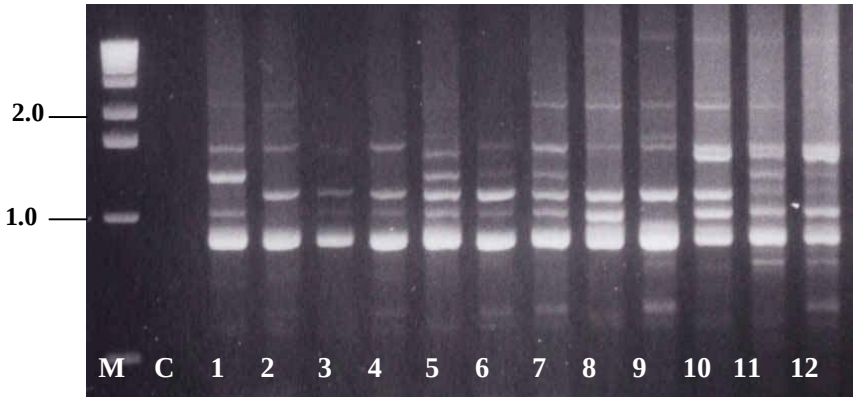


Figure 2: Phylogram of 12 Tunisian date-palm varieties constructed from Nei and Li genetic distance estimated from ISSR markers. Clustering with the UPGMA method