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**IDENTIFICATION OF *Phoenix dactylifera* L. VARIETIES BASED ON
AMPLIFIED FRAGMENT LENGTH POLYMORPHISM (AFLP)
MARKERS**

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Abstract: The amplified fragment length polymorphism (AFLP) technique was applied to identify palm varieties. Fluorescence labelled primers were used in selective amplifications and the amplified fragments were detected on capillary gel electrophoresis using an automated DNA sequencer with the analysis fragment option. This is a rapid and efficient technique for detecting a large number of DNA markers on the date palm. *Phoenix dactylifera* L. varieties Bou-Fegous, Medjool, and E-528 from Estación Phoenix (Elche), Spain, were analysed, yielding a total of 310 AFLP fragments derived from five primer combinations. The process for regenerating the date palm cultivars from *in vitro* tissue culture should yield individuals phenotypically and genetically identical to the explant they are derived from. The AFLP markers obtained were successfully used for comparing and identifying vitroplants of palm.

Key Words: *Phoenix dactylifera*, Amplified Fragment Length Polymorphism (AFLP), Molecular Markers, Vitroplant Analysis

INTRODUCTION

The date palm (*Phoenix dactylifera* L.) is a monocotyledoneous woody perennial belonging to the Arecaceae family, which comprises 200 genera and more than 2500 species [1]. Date palm breeding has relied and continues to rely on traditional methods. Advances in selection for agronomically important traits, such as fruit quality or disease resistance, are difficult due to the species long generation time.

Abbreviations used: AFLP – amplified fragment length polymorphism; RFLP – restriction fragment length polymorphism; RAPD – random amplification polymorphism DNA; SSR – simple sequence repeats; CTAB – hexadecyltrimethylammonium bromide; JOE – carboxy-4',5'-dichloro-2',7'-dimethoxyfluoresceine.

Genetic engineering and molecular markers have not thus far been used for the improvement of the date palm, but they are likely to play an important role in the future development of the crop. The clonal propagation of date palm (*Phoenix dactylifera* L.) elite cultivars with known performance would be of great benefit in countries where date palms are cultivated.

This long-lived dioecious monocotyledon has traditionally been vegetatively propagated from offshoots produced by individual trees. The conventional method is too slow and yields only a limited number of offshoots from a given parent tree. This means that *in vitro* tissue cultures would be an attractive alternative method for the mass propagation of the date palm.

Until recently, there was little study of crops at the genome level; however, the next few years should bring molecular breeding technology to this plant group. Molecular breeding can be defined as the application of DNA-based analysis of genome polymorphism to breeding programmes. Molecular markers can be linked to genes of interest, allowing indirect selection of the desired genotype. A second application of molecular marker technology is the identification of commercial varieties.

Technologies for genome fingerprinting (molecular marker detection) include restriction fragment length polymorphism (RFLP) [2], random-amplified polymorphism DNA markers (RAPD) [3], amplified fragment length polymorphism (AFLP) [4, 5] and simple sequence repeat polymorphism or microsatellites (SSR) [6-8].

The use of amplified fragment length polymorphism (AFLP) has some advantages in terms of use in the identification of diagnostic or specific markers. Although these markers are generally dominants, the AFLP technique does not require previous knowledge of the DNA sequence, generates reproducible fingerprinting profiles and allows the amplification of a high number of DNA fragments per reaction, enabling the detection of specific amplified fragments [4]. The genetic stability of *in vitro* derived clones is essential in the micropropagation of clones. For this purpose, it is necessary to develop molecular markers that make it possible to differentiate between genotypes. Traditionally, such characterization was achieved via disciplines like taxonomy and morphology, and using cytogenetic resources. With the AFLP technique, it is possible to analyze the genome of vegetal species.

The purpose of this study was to generate and improve DNA markers which could be used in AFLP studies for date palm cultivar identification, for genetic comparison and the identification of vitroplants obtained from adult palms.

MATERIALS AND METHODS

Plant material

Offshoots from five individuals of each date palm variety (Bou-Fegous, E-528 and Medjool) were collected randomly from plantations in Estación Phoenix in Elche, Alicante (Spain), and three vitroplants from the Medjool variety were

used in this study. The plant material was obtained from young leaves of plants grown in the field, and from vitroplants grown in culture media (for embryogenic calli).

The offshoot leaves surrounding the apical meristem were collected in liquid nitrogen, and stored at -80°C until processing for DNA isolation. The plant material was stored for several months, and no significant differences were found in the quality and quantity of the DNA isolated. Approximately 200 g of fresh material from plants or vitroplants was harvested. In the case of vitroplants, the entire plant was used.

DNA preparation

DNA was prepared from frozen leaves of adult trees and plants obtained by somatic embryogenesis. Total cellular DNA was extracted using the CTAB extraction method according to Dellaporta *et al.* [9], with the CTAB solution incubation times changed from 1 hour to 2 hours at 65°C .

AFLP procedure

The AFLP procedure was performed as previously described [4, 10] but was adapted for fluorescence detection following the AFLPTM Plant Mapping protocol [11]. Date palm DNA (500 ng) was double digested with *EcoRI* and *MseI*, and the resulting fragments were ligated to double-stranded adaptors. The sequence information of the adaptors is presented in Tab. 1.

Tab. 1. Oligonucleotide adaptors and primer combinations used for AFLP analysis.

Name	Sequence
EcoRI adaptor	5'-CTCGTAGACTGCGTACC-3' 3'-CTGACGCATGGTTAA-5'
MseI adaptor	5'-GACGATGAGTCCTGAG-3' 3'-TACTCAGGACTCAT-5'
Primers used in preamplification	
EcoRI + 1-A	5'-GACTGCGTACCAATTC + A-3'
MseI + 1-C	5'-GATGAGTCCTGAGTAA + C-3'
Primer combinations used in selective AFLP amplification	
EcoRI + 3- AAC *	5'-GACTGCGTACCAATTC + AAC -3'
MseI + 3- CAA *	5'-GATGAGTCCTGAGTAA + CAA -3'

* *EcoRI* Primer + **AAC**/AAG/ACA/ACC/ACG/ACT/AGC/AGG

* *MseI* Primer + **CAA**/CAC/CAG/CAT/CTA/CTC/CTG/CTT

The pre-amplification reactions were performed using 1 cycle of 2 minutes at 72°C , and 40 cycles, each cycle consisting of a 20 second DNA denaturation step at 94°C , a 30 second annealing step at 56°C , and a 2 minute extension step at 72°C . A final cycle was performed for 30 minutes at 60°C .

A 10 µl aliquot was removed from each sample and run on an 1.5% agarose gel to determine whether pre-amplification had been successful. The remaining volume was diluted 10-20 fold in water, according to the intensity of the smear, to adjust the samples to the equivalent DNA concentration.

Initially, 64 primer pairs were tested for selective amplification (Tab. 1). The *EcoRI* primer was labelled with a fluorescent group, carboxy-4',5'-dichloro-2',7'-dimethoxyfluoresceine (JOE).

Selective amplifications were performed with the following cycle profile: 1 cycle of 2 minutes at 94°C, 30 seconds at 65°C and 2 minutes at 72°C, followed by 8 cycles of 20 seconds at 94°C, 30 seconds of annealing in which the temperature was decreased by 1°C per cycle between 64°C and 57°C, and 2 minutes at 72°C, followed by 23 cycles of 20 seconds at 94°C, 30 seconds at 56°C and 2 minutes at 72°C. Starting at a very high annealing temperature allows for optimal primer selectivity. The efficiency of primer binding increases by gradually decreasing the annealing temperature. The amplification reactions were performed in a GeneAmp PCR System 2400.

The samples were prepared with deionized formamide and a GS-500 Carboxy-X-Rhodamine labelled size standard in each run in order to facilitate the automatic gel analysis and the sizing of the fragments. The samples were denatured by heating for 2 minutes at 95°C and analyzed via capillary electrophoresis using an ABI Prism 310 Genetic Analyzer. The lengths of the amplified fragments were determined by comparing them with the internal size standard, and the GeneScan software was used for analysis. AFLP fragments were scored manually for presence/absence for each sample on the electropherograms displayed.

AFLP patterns were determined in triplicate for each individual plant.

Data analysis

AFLP polymorphic bands were scored as present (1), absent (0) or a missing observation (-1) for the different genotypes. Often, several AFLP-markers within a primer combination show pleiotropic behaviour or very close linkage [12]. Likewise, in our set of genotypes, polymorphic markers with identical polymorphism patterns were found within some primer combinations. We also found markers within primer combinations that seemed to be allelic. In all of these cases, a second marker does not add any new independent information for a genetic-similarity estimate. Therefore, these redundant polymorphic markers within primer combinations were discarded before calculating genetic similarities.

Similarity matrices were constructed using the Dice coefficient [13]:

$$S_{ij} = 2a / (2a + b + c)$$

where S_{ij} is the similarity between two individuals, i and j , a is the number of bands present in i and absent in j , b is the number of bands present in j and absent in i . This definition of similarity excludes from the calculation bands that

are absent in both individuals, since mutual absence cannot necessarily be attributed to a common cause and therefore to similarity.

Conversion to the genetic distance D was obtained using the following equation:

$$D_{ij}=1-S_{ij}$$

Levels of diversity were estimated as the percentage of polymorphic bands out of the total scored.

RESULTS AND DISCUSSION

The AFLP technique is based on the selective PCR amplification of restriction fragments from a total restriction digest of genomic DNA [4]. The technique involves three steps: (1) restriction of the genomic DNA and ligation of oligonucleotide adaptors; (2) selective amplification of a subset of restriction fragments; and (3) gel-based analysis of the amplified fragments. The amplified restriction fragments are originated predominantly from unique loci on the genome, and are a source of DNA polymorphism. The differences in fragment lengths can be traced to base changes in the restriction site or in the primer extension site, or to insertions or deletions in the body of the DNA fragment. The number of bands that can be amplified in one AFLP reaction is very high, and it is normally necessary to tune the selective PCR amplification in order to select a band number that can be resolved by a gel electrophoresis system.

The fragments obtained for date palm AFLP were analyzed using fluorescent capillary electrophoresis, which delivers the required resolution, precision and analytical power for large-scale genomic fingerprinting, with the advantage of being more highly reproducible and faster than plain gel slabs [14]. The availability of a suitable DNA isolation procedure is a prerequisite for performing DNA-based marker studies on plant species. We tested several protocols [9, 15,16], and the best results were obtained with the method of Dellaporta – they gave DNA samples with A_{260}/A_{280} ratios between 1.6 and 1.9. Template DNA quantities were varied, and those fingerprints generated with less than 400 $\mu\text{g}/\text{ml}$ of template DNA were similar to the other fingerprints, although bands varied significantly in their intensity (peak heights) and some bands were absent. These results demonstrate that the AFLP procedure is not affected by the template DNA concentration, although aberrant fingerprints may be observed at very high template dilutions, giving only a few template molecules at the onset of the reaction. More probably, the individual restriction fragments are not randomly distributed at such low DNA concentrations, explaining the observed differences in band intensities. This is supported by our finding when comparing a number of individual AFLP fingerprints obtained with only 280 $\mu\text{g}/\text{ml}$ of template DNA; there was a high variation in the intensity of the individual bands. There are important differences between varieties in the quality and quantity of the DNA extracted; the quantity extracted from Medjool was two-fold higher than from Bou-Fegous.

The banding patterns for all the varieties tested were highly reproducible. Between two digestions and PCR runs, over 95% reproducibility was maintained.

In this study, we found primer combinations that yielded uniform distributions of the peaks in the region analysed from 130 bp to 360 bp, enabling us to use them as polymorphic markers. Fig. 1 shows representative electropherograms of fluorescent dye-labelled AFLP products, run on an ABI 310 Genetic Analyzer and analyzed using GeneScan analysis software.

The AFLP process generates fingerprint patterns of different fragment lengths that are characteristic and reproducible for an individual organism. The primer combinations used amplified different subsets of restriction fragment lengths resulting in several fingerprints.

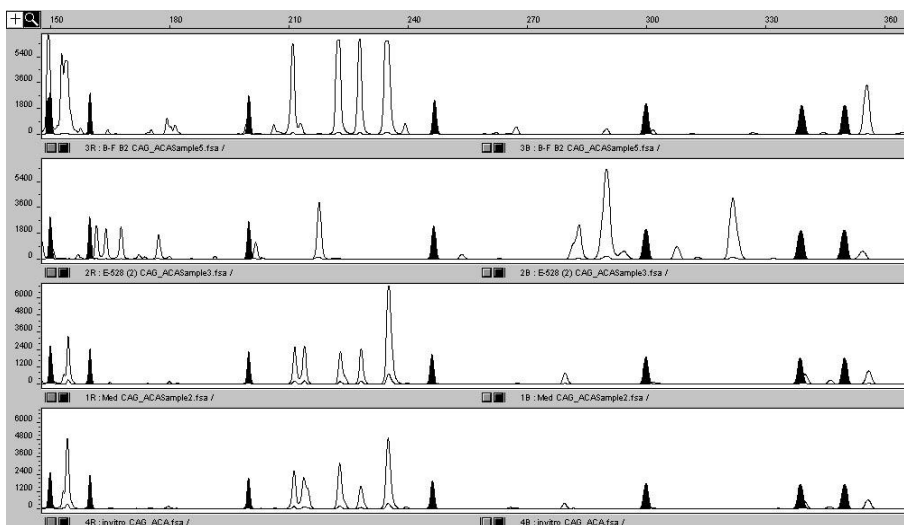


Fig. 1. AFLP fingerprints for *Phoenix dactylifera* L. varieties. From top to bottom: Bou-Fegous, E-527, Medjool and vitroplant from the Medjool variety. The DNA fingerprints were generated using *EcoRI* + *ACA*/*MseI* + *CAG*. The size markers are filled in black.

Not all the primer combinations revealed polymorphisms in each variety. Sixty-four primer combinations were tested in the three *Phoenix dactylifera* L. varieties analyzed. The genetic-similarity estimate was calculated following Nei and Li [17]. From all the patterns obtained, for intravariety comparison, we chose the primer combination corresponding to a lower similarity index (S_{ij}), because this means a high genetic distance (D_{ij}) between these varieties. The best results were obtained with five primer combinations that yielded 220 polymorphic bands of a total of 310, with an average percent of polymorphism of 72% between all the pairs (Tab. 2).

Tab. 2. The polymorphism rates between the palm varieties Medjool, Bou-Fegous, hybrid E-528.

Primer combinations	Total number of bands	Polymorphic bands	% Polymorphism
CAT/AAC	63	48	76
CAG/ACA	72	51	71
CAG/ACG	61	38	62
CTA/ACC	43	37	86
CAT/ACA	71	46	65
	total: 310	total: 220	mean: 72

Tab. 3. The primer combinations to compare the three varieties: Medjool, Bou-Fegous and hybrid E-528.

Primer combinations	Varieties	Polymorphic bands	Similarity index	Genetic distance
CAT/AAC	(M/B)	20	0.67	0.33
	(M/E)	25	0.21	0.79
	(B/E)	18	0.20	0.80
CAG/ACA	(M/B)	19	0.73	0.27
	(M/E)	25	0.28	0.72
	(B/E)	28	0.35	0.65
CAG/ACG	(M/B)	20	0.70	0.30
	(M/E)	20	0.46	0.54
	(B/E)	21	0.45	0.55
CTA/ACC	(M/B)	16	0.32	0.68
	(M/E)	17	0.21	0.79
	(B/E)	10	0.18	0.82
CAT/ACA	(M/B)	8	0.64	0.36
	(M/E)	24	0.36	0.64
	(B/E)	22	0.25	0.75

In Medjool, the five primer combinations displayed between 8 and 25 polymorphic bands. Other combinations amplified too many or too few fragments. In Bou-Fegous, the same combinations displayed between 10 and 28 polymorphic bands. The hybrid E-528, yielded between 10 and 25 polymorphic bands with these primer combinations (Tab. 3). These primer combinations yielded significant differences, allowing the genetic characterization. Tab. 3 shows the similarity index and genetic distance of the patterns selected. The genetic distance is enough to establishment a differentiation between the varieties of palms. Also, with these primer combinations, there are no differences within varieties.

The vitroplant study was performed with vitroplants obtained by somatic embryogenesis from Medjool adult plants. The DNA amount of the vitroplant assayed was 400 µg/mL. We tested if the primer combinations used for the identification of the adult plant varieties were also positive for vitroplants. The results showed that the AFLP procedure was highly reproducible with vitroplants, and that it allows comparison between adult plants and vitroplants. Fig. 1 shows the AFLP analysis of a vitroplant that belongs to the Medjool variety. With this technology, we are able to establish if a vitroplant variety is the same as that of an adult one or if it presents a different fingerprint. For plants obtained by somatic embryogenesis, it is necessary to perform a genetic analysis of vitroplants to test if a genetic modification has occurred in the process, in order to select the suitable vitroplants.

The AFLP technology is sensitive enough to detect low levels of variation, allowing us to discriminate between highly related varieties. Date palm cultivar identification by AFLP, RFLP and RAPD has been reported [18, 19], but this is the first paper on the development of AFLP DNA markers for date palm cultivars applied to the identification of vitroplants. With AFLP technology, cDNA libraries and radiolabelled probes are not needed. Moreover, in this study, fluorescence labelled oligonucleotides are employed in combination with automated capillary electrophoresis, giving a much higher resolution than with other AFLP detection systems.

The results presented here showed that AFLP can be successfully used for molecular marker testing in different palm varieties with a high resolution and reproducibility and allowing the identification of vitroplants. On this basis, it is possible to look for linkages between molecular markers and agronomically important traits, and also to identify genetic variation at different stages of the breeding process.

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