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CLONING DATE PALM (*Phoenix dactylifera* L.) DNA AND CHARACTERIZATION OF LOW, MEDIUM, AND HIGH COPY DNA SEQUENCES

Keywords: Date palm; genomic library; RFLP.

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*Total DNA was extracted from mature leaves of the date palm (*Phoenix dactylifera *L.) cultivar 'Bou-Sthammi Noire'. The DNA was digested with* Pst*I and the fragments shotgun-cloned in* E. coli *DH5α using the plasmid vector pUC19. A total of 124 clones were characterized by dot blot hybridization using ^{32}P labelled genomic DNA as the probe. 74% of the isolates contained single copy sequences. Many of the cloned fragments (58%) were less than 900 bp. Some of the single copy clones, when used as probes in Southern blot analysis of DNA extracted from twelve Moroccan date palm cultivars, detected restriction fragment length polymorphisms. The construction and the availability of a genomic library opens up the possibility of obtaining a panel of potentially interesting genomic DNA clones for initiating RFLP studies on breeding line of Moroccan date palms in order to speed up the selection and breeding of Bayoud resistant palms in Morocco.*

INTRODUCTION

An important date palm breeding programme was initiated by the Moroccan Institut National de la Recherche Agronomique (INRA), in the early 1970s. The ultimate goal of the project is to restore date palm groves devastated by the Bayoud disease caused by *Fusarium oxysporum* f. sp. *albedinis*. This pathogen threatens to destroy important date

palm groves in Morocco and other Maghreb countries such as Algeria and Tunisia. The use of DNA technology to develop suitable genetic markers is now urgently needed to speed up the progress of breeding in date palms.

However, date palm breeding has relied, and continues to rely, upon traditional methods based on the observation of morphological characters and the selection of useful traits on a purely phenotypic basis. Advances in selection for agronomically important traits, such as fruit quality or disease resistance, have therefore been slow because of the long generation time of this tree crop (Saaidi, 1992). Appropriate DNA markers have the potential to make significant contributions to date palm breeding crop improvement efforts by avoiding the long delay in identification of date palm cultivars based on adult phenotypes. For the breeder, variety and phenotype-specific markers can help identify and classify the different accessions in a germplasm collection and facilitate selecting desirable genotypes from extensive breeding populations. Secondly, with increasing research and the development of efficient micropropagation systems for elite resistant varieties of date palm (Aitchitt, 1989), genetic markers for determining the fidelity of *in vitro* propagated plants by a method applicable during different stages of tissue culture and in young plants in the nursery are also urgently needed.

Isoenzymes have been reported as potential biochemical markers in date palm selection and breeding (Torres and Tisserat, 1980; Baaziz and Saaidi, 1988; Bennaceur *et al.*, 1991). The main limitations of this technique are the lack of sufficient polymorphism between closely related cultivars and the fact that enzymes and other proteins may vary in different tissues according to developmental stages and the environment or conditions under which source plants are grown, because isoenzymes are products of gene expression (Beckman and Soller, 1986). DNA markers such as restriction fragment length polymorphisms (RFLPs) and random amplified polymorphic DNAs (RAPDs) have been developed to overcome such limitations and are now being widely used in genome mapping, fingerprinting of genotypes, and even as an aid in map-based cloning of agronomically important genes (Helentjaris, 1988; Hu *et al.*, 1991; Paran and Michelmore, 1993; Demeke *et al.*, 1993; Foolad *et al.*, 1993). We report our preliminary attempts to initiate DNA characterization methods for date palm following the development of a standardized procedure for extracting total date palm DNA from mature leaves (Aitchitt *et al.*, 1993).

We present here the result of successful cloning of date palm DNA and the characterization of low, medium and high copy sequences and their use as RFLP probes.

EXPERIMENTAL

Plant material

Mature leaves from the date palm cultivar 'Bou-Sthammi noire' were harvested from adult trees held in the INRA date palm germplasm collection at Zagora Experimental Station, Zagora, Morocco. This is one of the cultivars which is resistant to the Bayoud disease caused by *Fusarium oxysporum* f. sp. *albedinis*. Leaf samples were packed in plastic bags and transported by air freight on wet ice to Wye College and stored at –80°C until required for DNA extraction work.

Extraction and restriction of total date palm DNA

Total DNA from mature date palm leaves was extracted using a CTAB buffer as described by Aitchitt *et al.* (1993). 5µg aliquots of date palm DNA were digested at 37°C overnight with 20 units of *Pst*I enzyme in a 20 µl reaction volume containing the appropriate enzyme butter recommended by the supplier (BRL, UK). The quality of DNA and its digestion with the enzyme were analyzed by electrophoresis in 0.8% agarose gel of date palm DNA extracts run alongside a 1 Kb ladder marker (BRL, UK) at 40 V for 6 hours (*Figure 1*).

Recombinant DNA techniques

Methods used for cloning date were essentially as described by Sambrook *et al.* (1989). To increase cloning efficiency, the *Pst*I-digested pUC19 plasmid vector was dephosphorylated with calf intestinal alkaline phosphatase. The recombinant plasmids were selected by transforming competent *E. coli* cells (strain DH5α) which were prepared using the $CaCl_2$ method (Sambrook *et al.*, 1989).

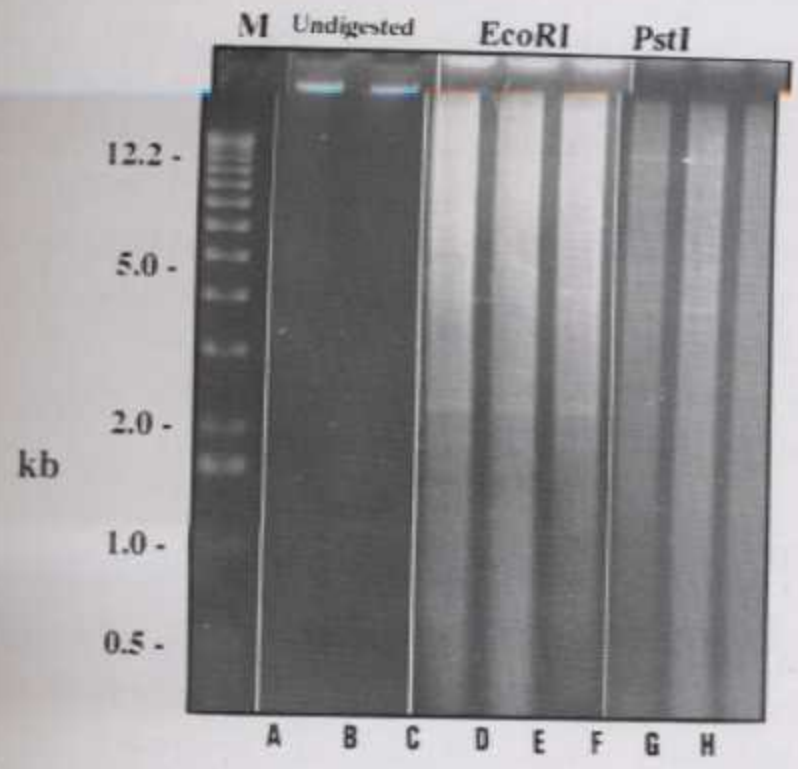

Figure 1. Electrophoretic analysis of date palm DNA. Aliquots of undigested (1µg) and digested DNAs (5µg of DNA digested with 20 units of enzymes for 12 h) from different cultivars of date palm were analyzed on 0.8% agarose gel in TAE. The undigested DNA samples are from Bou-Sthammi Noire (lane A) and Bou-Feggous (lane B) and EcoRI (lanes C–E) and PstI (lanes F–H) digests of Bou-sthammi Noire, Bou-Feggous and Jihel respectively. The 1 kb ladder (BRL, lane M) was used as the size marker.

Screening for recombinant clones

Transformants were selected on LB media plates containing 100 µg/ml X-gal (5-Bromo-4-chloro-3-Indolyl-beta-D-galactopyranoside) and 1 mM of IPTG (Isopropylthio-β-D-galactoside). Potential recombinants were identified initially by the blue/white selection (whites are recombinants by contrast to the blues). The putative recombinants were then screened by a standard colony lysis procedure (Barnes, 1977) and recombinants were identified by the reduced mobility in comparison with a control (vector) plasmid (*Figure 2*). Isolates containing plasmids with significantly reduced mobility were grown at 37°C in 10 ml of LB medium (in universal flasks) containing ampicillin (100 µg/ml) and cultured for 16 hr with shaking (300 rpm). Plasmid DNAs were prepared by the boiling lysis procedure of Holmes and Quigly (1981). A 10 ml culture generally yielded 50–100 µg of plasmid DNA. Such preparations were sufficiently pure and could be used directly in restriction digestion reactions to determine insert sizes and purify inserts and for DNA sequencing (*Figures 3a* and *b*). The DNA concentrations of the individual DNA extracts were estimated after their separation by electrophoresis on agarose gel and comparison with DNA concentration markers.

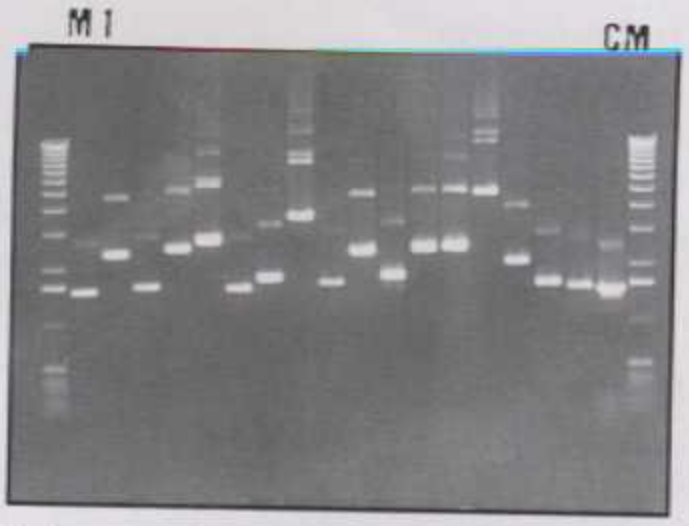

Figure 2. Agarose gel electrophoresis of recombinant plasmids containing date palm DNAs. Colony lysates from potential recombinants were analyzed on a 0.8% agarose gel in TAE. Plasmids with no or very small insert (e.g. lane 1) had a similar mobility as compared to pUC19 DNA (lane C). The recombinant plasmids with significantly large inserts migrated more slowly (lanes 2–17). The presence and sizes of the different inserts were confirmed after digestion with PstI (Figure 3). Lane M is the 1 Kb DNA ladder (BRL).

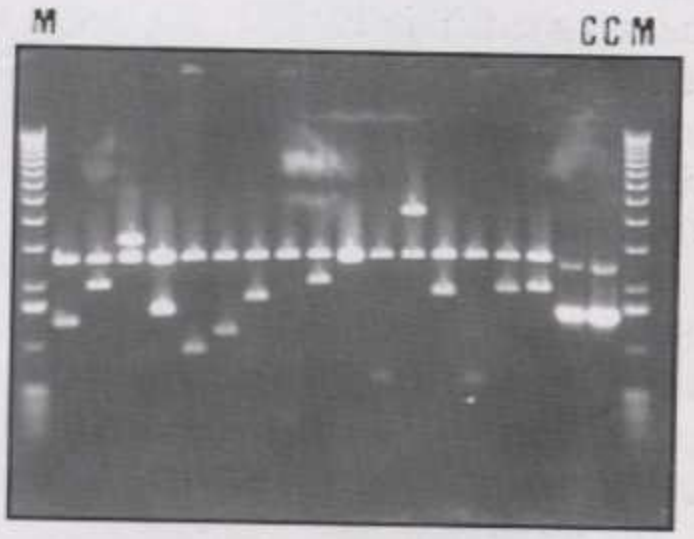

Figure 3a.

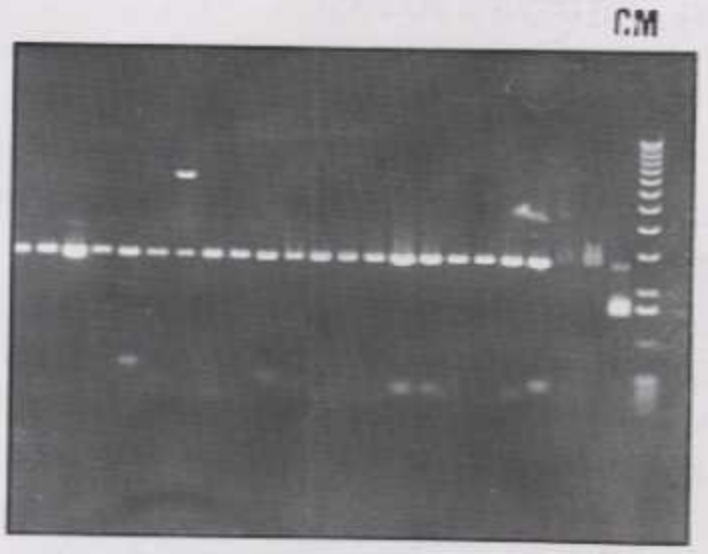

Figure 3b.

Electrophoretic analysis of PstI digested recombinant plasmids. Inserts of date palm DNA in recombinant clones were released from the vector following restriction digestion with PstI. The sizes of the insert were estimated by comparison to DNA markers (1 kb DNA ladder, BRL) which was electrophoresed in parallel lanes (M). pUC19 plasmid DNA was the control (lane C). The inserts ranged from < 500 bp (the smallest) to ca. 5.5 kb (the largest) (Figure 3b).

Dot Blot Hybridization

The copy number status of the cloned DNAs within the cv. 'Bou-Sthammi Noire' genome was estimated by a dot blot hybridization procedure using ^{32}P-labelled total DNA as the probe. A 1µg aliquot of recombinant plasmid DNA was denatured in 100 µl of 0.4 M sodium hydroxide at 37°C for 20 min. Then 4µl (approx. 40 ng) of of the alkali-denatured DNA solution and 4 µl (approx. 4 ng) of 1/10 dilution of the same preparation were spotted adjacent to each other on a nylon membrane (Hybond N$^+$, Amersham UK). The two different dilutions were used in order to exclude errors from denaturation and/or hybridization artefacts. The membrane was prehybridized for 4 hr at 65°C in 6 × SSC (1 × SSC contained 0.15 M NaC1, 15 mM sodium citrate, pH7.0), 5 × Denhardt's solution (100 × Denhardt's solution is 2% PVP, 2% bovine serum albumin and 2% Ficoll), 0.1% SDS and 10µg/ml denatured salmon sperm DNA. The membrane was hybridized for 16 hr in the same buffer containing 5 × 10^5 cpm/ml ^{32}P labelled 'Bou-Sthammi Noire' DNA. The membrane was then initially washed in 3 × SSC/1% SDS at 65°C (15 min) followed by a second wash with 2 × SSC/1% SDS (15 min). The filters were then monitored and washed further, if necessary, with 0.2 × SSC/1% SDS for 2 minutes at 65°C. The membrane was wrapped in Saran and exposed to Kodak X-OMAT AR autoradiography film between intensifying screens at –70°C.

RESULTS AND DISCUSSION

Isolation of high molecular weight DNA from date palm leaves

Using the simple method described previously by Aitchitt *et al.*, (1993) total DNA was extracted from mature date palm leaves. Agarose gel electrophoresis confirmed that the DNA was of high molecular weight with no degradation or contaminating RNA. Restriction analysis also showed that such DNA preparations were of sufficient purity to act as a good substrate for restriction enzymes (*Figure 1* shows the profile of the digestion products with *Pst*I and *Eco*RI). The presence of intermittent strong bands resulting from digested repetitive DNAs was used as a visual indicator of the quality of a digestion (the completeness of digestion and the integrity of restriction fragments). The staining intensities of these band suggested minimal degradation of digestion products. These restriction fragments, which were obtained by digesting DNA isolated using the CTAB procedure, could also be easily cloned in pUC 19 and a very large number of recombinants could be recovered.

Characterization of recombinant clones

In order to study the characteristics of such a randomly cloned *Pst*I library a small sample of 124 recombinants was selected for analysis. The restriction analysis of the recombinant plasmid DNAs showed that the cloned fragments ranged from as little as, or smaller than, 500 bp to 5.5 kb. A large proportion of the inserts were of small sizes: 58.3% were < 900 bp, 31.3% were of 1–2 kb, and a small proportion (3.8%) were > 3.5 kb with a maximum size of 5.5 kb (*Figures 3a* and *b*). Inserts > 900 bp (41.7% of the total) were easily visualized on an agarose gel and purified from it for subsequent use as probes (*Figure 4*). The yield of purified inserts smaller than this size was low, but large amounts of such small fragments could be amplified by the polymerase chain reaction (PCR) using M13 reverse and forward primers.

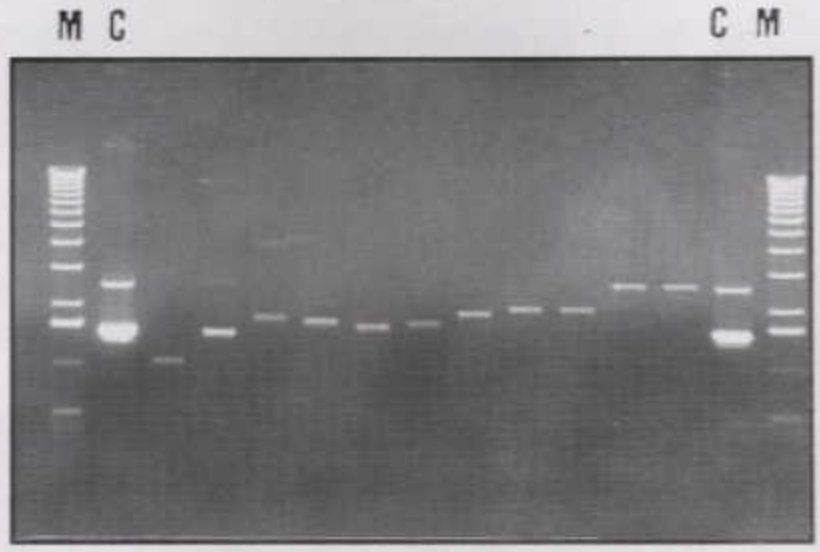

Figure 4. Cloned and purified palm DNA fragments. The inserts released from the pUC19 vector (shown in Figure 3*) were separated by agarose gel electrophoresis and purified using the Qiagen kit (Qiagen, USA). An aliquot of the purified insert was reloaded in 0.6% agarose gel to check the recovery, size and integrity of DNA before using as RFLP probes. pUC19 (lane C) was used as control.*

Choice of *PstI* as the cloning enzyme and enrichment of low copy DNAs

*Pst*I is a methylation-sensitive restriction enzyme (Nelson and McClelland, 1987; Backman, 1980). Transcribed or transcriptionally active DNAs are known to be non-methylated or hypomethylated by comparison with the hypermethylated inactive regions. Therefore *Pst*I digestion of genomic DNAs avoids fragmenting the heavily methylated and repeated regions of the genome. Consequently, clones derived from *Pst*I restriction of fragments are more likely to represent transcriptionally active regions of the genomes and hence single or low copy sequences. This approach has been recommended for the construction of libraries (Smith and Smith, 1992) and has been successfully used to enrich low copy number sequences for use as DNA probes for RFLP analyses of different plants including palms (*e.g.* oil palms, Dr P. Jack and colleagues, personal communication, Shah and Parveez, 1992). Our experiments with date palm DNA also showed a similar enrichment of low copy sequences in the 124 independent clones which were isolated and characterized (see below).

The cloned DNA could be shown to be derived from date palm by hybridizing it to ^{32}P-labelled high molecular weight total date palm DNA. Based on the intensity of hybridization it was also possible to group the cloned DNAs arbitrarily, according to their copy number in the date palm genome, into low, medium or high copy sequences (*Figure 5*). The artefacts resulting from unequal amounts of plasmid DNA or uneven denaturation and unequal hybridization were discriminated by using dilutions of the stock-denaturated DNAs. These results (not shown) also confirmed the preliminary hybridization results. Of the 124 clones screened, 74.3% were single copy, 14.3% medium copy and 11.4% repetitive sequences. The relatively high number of single copies obtained was expected because of the restriction endonuclease chosen to construct the library. The repeated clones represent either DNAs from the nuclear genome or DNAs from the highly repeated plastid or mitochondrial chromosomes. Some of the selected clones were also end-sequenced and used to construct primers employed in a detailed study on amplified DNA polymiophisms (details presented in Aitchitt, 1994).

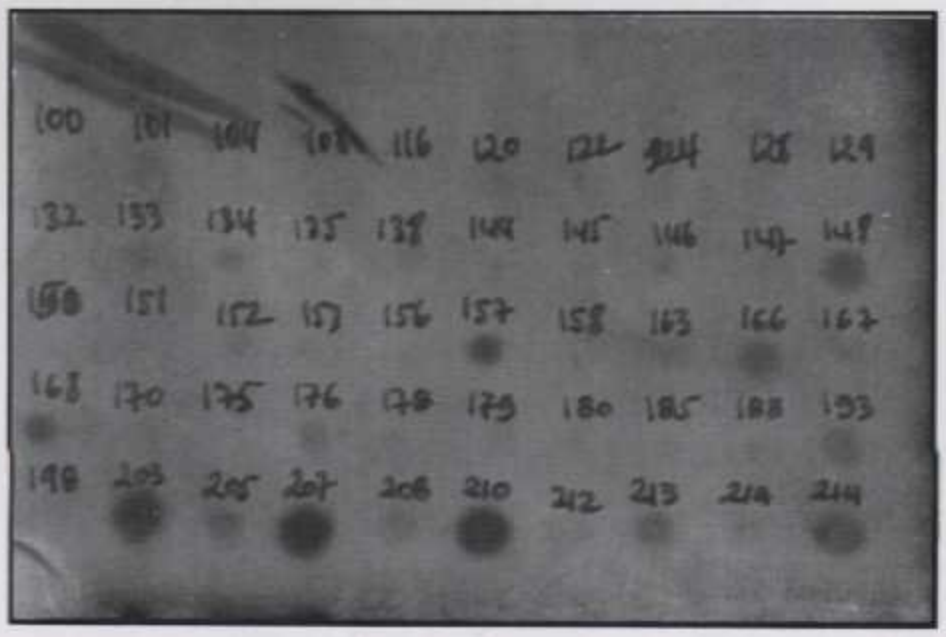

Figure 5. Analysis of recombinant clones by dot blot hybridization. Recombinant plasmid DNAs were denatured with NaOH and approximately 40 ng (in 4µl) was spotted on Hybond N⁺ and hybridized to ^{32}P-labelled total genomic DNA. Autoradiography confirmed that the cloned sequences are derived from the date palm genome. The observed differences in the intensities of hybridization were indications of the copy number status of the cloned DNA within the genome [high; e.g. clone 203 or 207; medium, e.g. clone 213 and low; e.g. clone 128 or 129]. In order to eliminate hybridization artefacts these results were also confirmed with dilutions of plasmid DNA (data not shown).

The results presented here show, first of all, that the DNA purified from a resistant cultivar or date palm is a good substrate for *Pst*I digestion and that the resulting restriction fragments can be successfully cloned in plasmid vectors; secondly, the cloned fragments obtained were of variable size but their size distribution was skewed towards a large number of small fragments; thirdly, as expected from the choice of the enzyme (*Pst*I), a very high proportion of fragments consisted of single copy DNAs. Polymorphisms could also be detected in a preliminary RFLP study (*Figure 6*).

Because these were pilot experiments, we did not attempt to maximize the cloning of DNA fragments. This could have been done by increasing the efficiency of transformation of bacterial cells (*e.g.* by electroporation). The skewed distribution and the unusually large proportion of smaller fragments in the above experiment could be rectified by fractionating the restriction fragments by electrophoresis or by sucrose gradient centrifugation prior to cloning. These extra steps should also help in obtaining a more representative and larger collection of DNAs for use as probes in RFLP studies and for other applications in future experiments which are

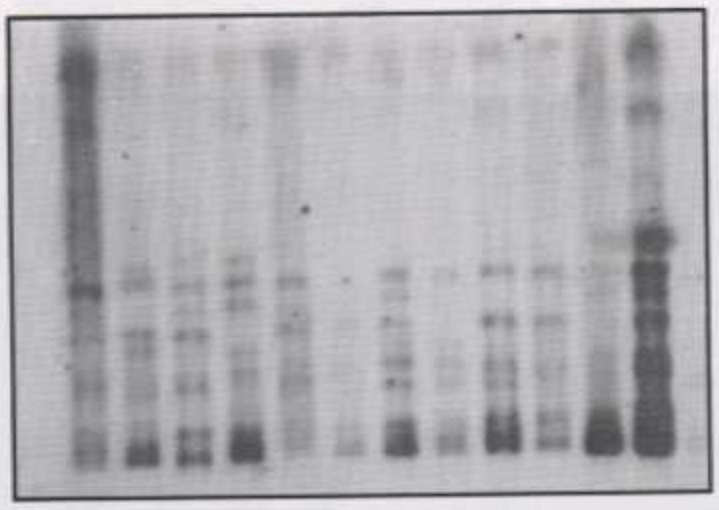

Figure 6. RFLP analysis of 12 Moroccan cultivars of date palm. 5 µg aliquots of high molecular weight DNA from mature leaves of 12 Moroccan cultivars were digested with EcoRI, fractionated on a 0.8% agarose gel, transferred to Hybond N+ (Amersham, UK) and probed at high stringency conditions with the insert from isolate 128, a putative single copy clone. The probe detected polymorphism within the different cultivars used, gave an easily scorable pattern and proved the usefulness of the cloned date palm DNAs for such analyses. The description of a detailed RFLP analysis is in preparation.

planned. Enriching for nuclear DNAs (Watson and Thomson, 1988) or eliminating mitochondrial or chloroplasts sequences will also be beneficial for obtaining probes that will detect polymorphisms specifically within the nuclear genome of the date palm. The availability of such a panel of homologous and potentially single copy DNA probes will enable very high stringency conditions for RFLP analyses of date palms.

Detection of RFLP by genomic DNA probes

In order to test if it was possible to detect a polymorphism between different Moroccan date palm varieties, a preliminary screening of different combinations of restriction enzyme and probe was carried out. Genomic DNA extracted from mature leaves of different date palm varieties was digested with different restriction enzymes according to the manufacturers' instructions. Restricted DNA fragments were separated by electrophoresis on 0.8% (w/v) agarose gels in TAE buffer and then blotted with 10 × SSC on to Hybond N⁺ membrane (Amersham) following the standard procedure (Sambrook *et al.*, 1989). The filters were then UV-cross linked and hybridized. DNA probes purified from agarose gel were labelled with ^{32}P-dCTP by the random primer method according to the manufacturers' instructions, using a Pharmacia kit. Nylon membranes were prehybridized for 4 hr at 65°C in 6 × SSC, 5 × Denhardt's solution, 0.1% SDS and 100µ ml^{-1} denatured salmon sperm DNA, then hybridized for 16 hr in the same butter containing 5×10^5 cpm ml^{-1} ^{32}P-labelled probe. The membranes were washed in 0.2 × SSC and 1% SDS for 2–4 min if necessary. Signals from such filters were detected by autoradiography. A representative auto-radiograph is shown in *Figure 6*. The variety No. 14 is unique in its restriction fragment profile. More probes which detect such variety specific restriction patterns are being identified.

CONCLUSION

The results presented in this report show first of all that the DNA purified from a resistant cultivar of date palm is a good substrate for *Pst*I and the resulting restriction fragments can be successfully cloned in plasmid vectors. The cloned fragments were of variable sizes but were predominantly small fragments (58% were smaller than 900 bp). The choice of *Pst*I resulted in a very high proportion of single copy sequences. The sequences were used to detect polymorphisms in RFLP studies (*Figure 7*). The use of such DNA markers in date palm breeding will speed up the progress of the improvement of this species for economic traits such as high yield, fruit quality and resistance to disease, especially Bayoud (*Fusarium oxysporum* f. sp. *albedinis*), which is the biggest threat to the date palm in North Africa.

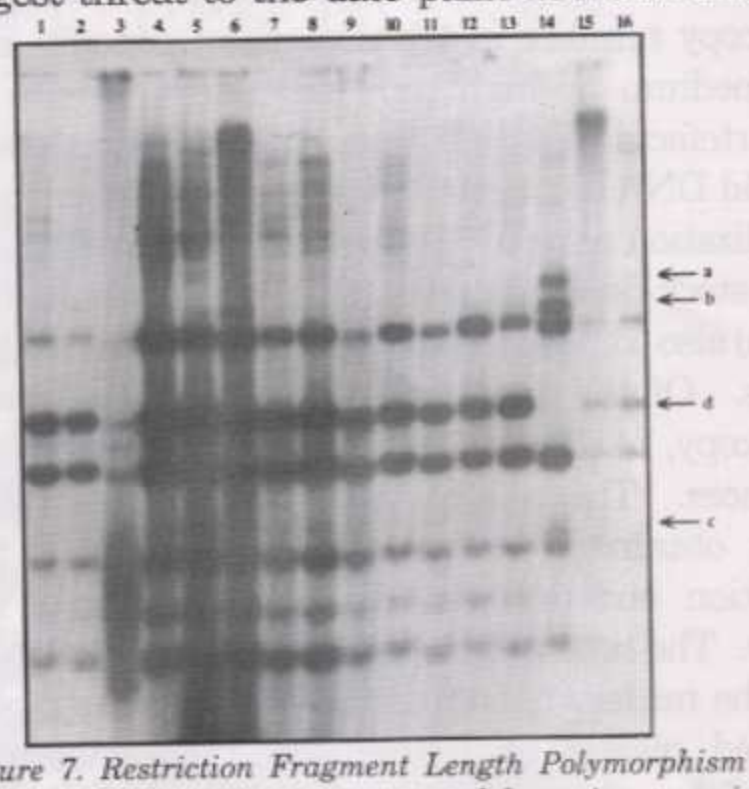

Figure 7. Restriction Fragment Length Polymorphism in date palm. Genomic DNA (10µg) extracted from sixteen varieties was digested with SSt *I (50U) tranferred to Hybond N⁺ (Amersham) and probed with ^{32}P labelled 1.2 kb P:tI fragment (clone 128, low copy). A distinct polymorphism is noticed in varie:" No. 14 (loss of one band 'd' and gain of three 'a', 'b', 'c').*

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