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Identification and genetic diversity analysis of date palm (*Phoenix dactylifera* L.) varieties from Morocco using RAPD markers

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Summary

Genetic variation among 43 date palm (*Phoenix dactylifera* L.) accessions, including 37 accessions from Morocco and 6 cultivars from Iraq and Tunisia, was studied using Random Amplified Polymorphic DNA (RAPD) markers. The pre-screening of 123 primers on four genotypes allowed selection of 19 primers which revealed polymorphism and gave reproducible results. All 43 analysed genotypes were distinguishable by their band patterns. RAPD technology therefore appears very effective for identifying accessions of date palm. RAPD-based genetic distance was used to determine the relationships between the accessions. The grouping-association identified by cluster analysis was rather weak. However, morphologically similar varieties clustered together. A relatively low polymorphism and a lack of evident organisation are observed among the date palm varieties grown in Morocco. This could be related to the mode of introduction and maintenance of the Moroccan date palm germplasm involving limited foundation germplasm, exchange of cultivars between plantations, and periodic development of new recombinant cultivars following sexual reproduction.

Introduction

Date palm (*Phoenix dactylifera* L.) is one of the earliest cultivated tree crops (Wrigley, 1995). It is only found as a cultivated plant, in abandoned gardens, or at desert waterholes where it has grown from seed discarded by travellers. It is believed to be a native of the Arabian Gulf region, possibly southern Iraq. In very early times, the date palm was introduced by man in northern India, North Africa and southern Spain, where it plays a major role in arid zones. In the oases of Morocco, for instance, the cultivation of date palm constitutes by far the main income-generating activity.

Despite its outstanding agronomic and socio-economic significance, attempts to improve knowledge and use of date palm biodiversity have been limited and are becoming an urgent priority. In particular, date palm plantations in North Africa are currently in danger of being destroyed due to a severe wilt caused by *Fusarium oxysporum* f. sp. *albedinis* and called 'Bayoud' (Pereau-Leroy, 1958; Brac de la Perrière & Benkhalifa, 1995). Tolerant or resistant Moroccan cultivars have already been identified but, unfortunately, they all give poor to medium date quality (Louvet & Toutain, 1973; Saaidi et al., 1981). The Irakian and Tunisian cultivars evaluated so far for resistance to Bayoud appeared to be susceptible in field conditions. Trees called 'khalt', which recover from seedlings following sexual reproduction, present a large amount of polymorphism due to the high heterozygosity of parental varieties. Khalts have been observed in plantations to be resistant and to present a good fruit-quality (Djerbi et al., 1986; Sedra, 1992, 1995; Sedra et al., 1996). Characterisation and analysis of the available genetic diversity therefore constitute an indispensable step with regard to the development of breeding strategies.

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The date palm is a dioecious (i.e. cross-fertilisation obligatory) perennial monocotyledon with long generation times (a period of 4 to 5 years is necessary to reach the first flowering). It has traditionally been vegetatively propagated from offshoots produced by elite individual trees. In Morocco, more than 220 clonally propagated varieties are known (Toutain et al., 1971). All commercial varieties are female and there is no method yet of producing male palms of these varieties. However, the effects of pollen on date quality through metaxenia are well documented, and male genotypes with desirable qualities are maintained in the plantations and commonly used to hand pollinate female trees.

Correct identification of trees is usually not possible until fruit are produced. In addition, the characterisation of cultivars and evaluation of genetic diversity require a large set of phenotypic data that are often difficult to assess and sometimes variable due to environmental influences (Sedra et al., 1993, 1996). Isoenzyme markers have proved of some use in cultivar identification (Baaziz & Saaidi, 1988; Bennaceur et al., 1991; Fakir et al., 1992; Bendiab et al., 1993). However, they are limited by the number of informative markers and give no direct assessment of the DNA genomic variation. Restriction fragment length polymorphisms (RFLPs) have been evaluated for date palm clone identification (Corniquel & Mercier, 1994), but the technique is laborious and not suited to studies of a large number of samples. Randomly amplified polymorphic DNA (RAPD) markers (Williams et al., 1990; Welsh & McClelland, 1990) are of particular interest. DNA profiles based on arbitrary primed PCR are both time- and cost-effective.

In the present study, we tested the reliability of the RAPD-PCR system as a tool for the identification of date palm cultivars. Furthermore, we sought an indication of the level of genetic variation and genetic relationships within the date palm cultivars grown in Morocco.

Materials and methods

Plant materials

The plant material consisted of 31 commercial varieties, 10 'khalts' originating from various plantations and selected for their date quality (Sedra, 1992, 1995), and 2 male genotypes. The commercial varieties comprised 3 cultivars from Tunisia, 3 cultivars from Iraq and 25 Moroccan cultivars. The most common Moroccan varieties and the main plantation areas of Morocco (Toutain et al., 1971) were represented. The analysed varieties present great morphological diversity for agronomic traits (Sedra et al., 1993, 1996). Further information on the materials is given in Table 1. All plant material was obtained from the field-collection of INRA-Morocco in Zagora.

DNA extraction and amplification

Genomic DNA of each genotype was extracted from 4 g of lyophilised leaflets. The leaves were first ground into fine powder. DNA was extracted in 200 ml of extraction buffer (350 mM sorbitol, 100 mM Tris-HCl pH 8, 5 mM EDTA pH 8, 0.5% sodium bisulphate) and the solution was filtered through a muslin cloth. The extract was centrifuged at 3000 g for 20 minutes, and the supernatant discarded. The precipitate was incubated in 30 ml of lysis buffer (1.5 M NaCl, 100 mM Tris-HCl pH 8, 20 mM EDTA pH 8, 4% mixed alkyltrimethylammonium bromide) for 4 hours at 65 °C with occasional mixing. After cooling for about 5 minutes at room temperature, the extract was adjusted to 50 ml by adding chloroform/isoamyl alcohol (24/1 v/v). The mixture was then homogenised by gentle inversion before being centrifuged at 3000 g for 10 minutes. The aqueous supernatant was recovered and the chloroform/isoamyl alcohol extraction procedure repeated. The resulting aqueous fraction was incubated with 100 μl of 10 mg/ml RNase (Boehringer Mannheim) for 30 minutes at 37 °C before precipitating the DNA with an equal volume of isopropanol. The precipitated DNA was recovered in 1 ml of 70% (v/v) ethanol, dried and dissolved in 300 μl of TE buffer (10 mM Tris-HCl, 1 mM EDTA pH 8). Depending on the leaf samples, yield in DNA varied from 40 to 400 μg using this protocol.

A total of 123 random decamer primers (Operon Technologies, CA, USA) were used for PCR in reaction conditions similar to those described by Lashermes et al. (1993). The RAPD products were fractionated according to size on agarose gel (1.8% w/w) subjected to electrophoresis (10 V/cm for 4.5 hours) in 1X TBE buffer. The DNA fragments were uniformly stained in a solution of ethidium bromide (10 μg/ml) for 15 minutes. DNA was visualised on a UV transilluminator and photographed using Polaroid film. Fragment length was estimated by comparison with standard size markers (λ phage double digested with *Hind*III and *Eco*RI).

Table 1. Name, origin, and main characteristics of date-palm genotypes studied

Code	Name	Geographical distribution*	Date characteristics				Resistance to 'Bayoud'**
			Appearance	Colour	Consistency	Maturity	
BSTN	Boustammi noire	A, D, F, G	Poor	Black	Soft	Late	R
BSTB	Boustammi blanche	D, G	Poor	Brown	Soft	Season	R
TDMT	Tademainte	A, F, G	Medium	Black	Semi-dry	Season	R
IKL	Iklane	A, D, F, G	Poor	Black	Soft	Very late	R
SLY	Saîrlayalate	D	Medium	Pale brown	Semi-dry	Late	R
BFGM	Boufeggouss/Moussa	D	Poor	Black	Soft	Early	R
HFS	Hafs	A, B, E	Poor	Black	Soft	Season	S
BSL	Bouslikhéne	B, F	Poor	Pale brown	Semi-dry	Early	MR
BZG	Bouzeggar	A, I, J	Poor	Black	Semi-soft	Very late	MR
AIB	Aîssa-Iyoub	E	Good	Pale brown	Semi-dry	Late	S
BHZ	Belhazit	B	Medium	Pale brown	Semi-soft	Season	S
AZO	Azigzao	B, G	Poor	Pale brown	Semi-dry	Early	MR
BIT	Bouittob	D, G	Medium	Pale brown	Dry	Late	S
ADM	Ademou	B	Good	Pale brown	Dry	Season	S
BIJ	Bouijou	K	Medium	Pale brown	Dry	Season	S
OTK	Otokdime	H	Medium	Pale brown	Dry	Late	MR
AGL	Aguélid	A	Poor	Pale brown	Semi-soft	Very early	MR
HOA	Houa	B	Medium	Pale brown	Semi-dry	Season	S
AHD	Ahardane	A, D, E, F, G	Medium	Brown	Semi-soft	Very early	HS
BKN	Boukhanni	A	Medium	Brown	Semi-soft	Early	R
MJH	Mejhoul	B, C	Excellent	Brown	Semi-soft	Very late	HS
BFG	Boufeggouss	All regions	Good	Brown	Soft	Season	HS
JHL	Jihel	A, D, F, G	Good	Pale brown	Dry	Very late	HS
BSK	Bouskri	A, D, F, H	Medium	Brown	Dry	Late	HS
BRR	Bourar	A	Good	Brown	Semi-dry	Late	S
DN	Deglet Nour	Tunisia	Excellent	Pale brown	Semi-soft	Season	HS
FTM	Ftimi	Tunisia	Medium	Pale brown	Semi-dry	Late	S
BSRL	Besser Lahlou	Tunisia	Medium	Pale brown	Semi-soft	Season	S
BARH	Barhi	Iraq	Good	Pale brown	Semi-soft	Early	HS
ZAHD	Zahdi	Iraq	Good	Pale brown	Semi-soft	Season	HS
HALW	Hallaoui	Iraq	Good	Pale brown	Semi-soft	Season	HS
SH-1	SH-1001	A	Good	Pale brown	Semi-soft	Early	R
SH-2	SH-1002	C	Excellent	Pale brown	Semi-dry	Early	R
SH-3	SH-1003	A	Very good	Pale brown	Semi-dry	Very early	R?
SH-4	SH-1004	A	Excellent	Pale brown	Semi-soft	Early	R?
SH-5	SH-1005	A	Very good	Pale brown	Semi-soft	Season	R?
SH-6	SH-1006	A	Very good	Pale brown	Semi-soft	Early	R?
SH-7	SH-1007	A	Very good	Pale brown	Semi-soft	Season	R?
SH-8	SH-1008	A	Very good	Pale brown	Semi-soft	Season	S
SH-9	SH-1009	A	Very good	Pale brown	Semi- soft	Season	S
SH-10	SH-1010	A	Very good	Pale brown	Semi-soft	Season	R?
NP3	NP3 (male palm)	A	–	–	–	–	R?
NP4	NP4 (male palm)	A	–	–	–	–	R

* Regions of Morocco: A = Drâa, B = Tafilalet, C = Ziz, D = Bani, E = Oriental, F = Saghro, G = Anti-Atlas, H = Todra, I = Ferkla, J = Ghéris, K = Guir.
** Resistance phenotypes: HS = Highly susceptible, S = susceptible, MR = Medium resistant, R? = Presumed resistant, R = resistant.

Table 2. Selected Operon primers with the numbers of amplified products and polymorphic fragments

Primers	Amplified products	Polymorphic fragments
OP-D3	4	1
OP-D4	2	1
OP-D10	2	1
OP-D12	5	3
OP-D15	2	2
OP-D16	3	3
OP-D19	2	1
OP-J4	2	1
OP-J5	3	3
OP-J13	3	3
OP-J14	4	2
OP-J18	2	1
OP-J19	4	3
OP-L6	5	2
OP-M5	2	1
OP-M11	6	4
OP-N1	2	2
OP-N12	1	1
OP-X4	2	2

Data analysis

Data were recorded as presence (1) or absence (0) of amplified products. Genetic distances (GD) between genotypes were estimated as follows: GDxy = (Nx + Ny)/(Nx + Ny + Nxy); where Nx is the number of bands in line x and not in line y, Ny is the number of bands in line y and not in line x, and Nxy is the number of bands in lines x and y. A distance matrix between the 43 accessions was constructed. Cluster analysis by the unweighted pair group method using arithmetic averages (UPGMA) was performed with the TREECON (version 1.1) software package (Van der Peer & De Wachter, 1993). In addition, a correspondence analysis (Benzecri, 1973) was performed using an NDMS (ORSTOM, France) statistical software package. A second clustering analysis using the 'Ward aggregation method' was performed based on the 4 main factor scores.

Results and discussion

Initially, the RAPD technology was applied to four genotypes (i.e. BSTN, ADM, ZAHD and AIB) representing different geographical origins. The number of amplification bands per primer varied between 0 and 13, with a mean of 5 major bands per primers. Of the 123 primers tested, 19 were selected for further analysis based on the intensity, size and number of amplified products (Table 2). To ensure reproducibility and genetic pertinence of RAPD marker data, the primers generating no, weak or complex patterns were discarded.

Analysis of the 19 selected primers among the 43 accessions included in this study generated 56 bands, 37 of which were polymorphic. There were 1.9 polymorphic bands per primer on average. Examples of polymorphism are shown in Figure 1. All 43 accessions were distinguishable by their band patterns. RAPD technology appears very effective for identifying accessions of date palm, although the overall exhibited polymorphism is rather low in comparison with results reported for other cultivated species (Hu & Quiros, 1991; Mossler et al., 1992; Yang & Quiros, 1993; Koller et al., 1993; Wolff & Van Run, 1993). RAPD-markers should therefore be of high value for date palm germplasm characterisation and genetic maintenance. Previous molecular markers studies (Aît Chitt et al., 1995; Corniquel & Mercier, 1994) involved a restricted set of date palm varieties and were less rewarding.

The 37 RAPDs were used to estimate relationships among the accessions. Genetic distance between accessions varied from 0.13 to 0.77. A dendrogram constructed by cluster analysis using RAPD-based genetic distance is presented in Figure 2. The overall tree topology suggested a rather weak grouping-association. However, morphologically similar varieties such as Boukhanni and Bouzeggar, as well as Boustammi noire and Tademainte, clustered together. As expected from their origin through sexual reproduction, the khalts showed a large diversity and, with the exception of SH7, differed from the varieties. SH7 was very similar (i.e. GD = 0.13) to the variety Iklane, which is grown in the date-plantation (Drâa) where SH7 was selected. Cultivars from Tunisia and Iraq did not exhibit a separation from the Moroccan germplasm. Furthermore, the cultivars resistant to the 'Bayoud' appeared in three different main groups. The male genotypes did not show distinct genetic differences from the female cultivars.

Results from the correspondence analysis are summarised in Figure 3. Major groups cannot be easily identified. However, relationships were similar to the UPGMA cluster analysis. It is notable that most of the cultivars from Tunisia or Iraq were associated

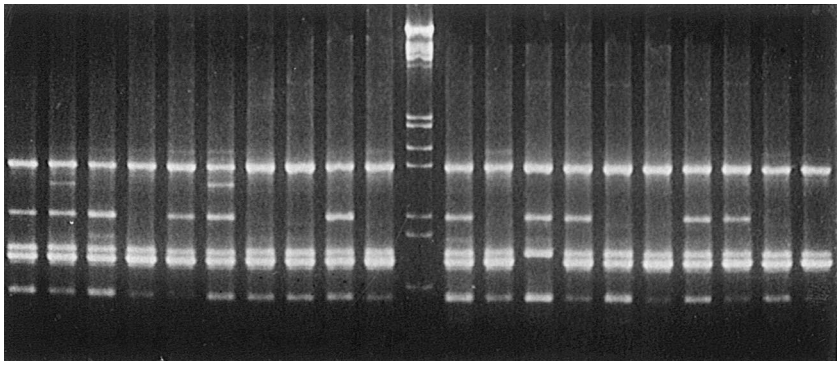

Figure 1. Examples of DNA polymorphisms detected between accessions of date palm. Ethidium bromide-stained agarose gel of amplification fragments produced with primer OP-M11. Lane 11 contains fragments of molecular weight markers.

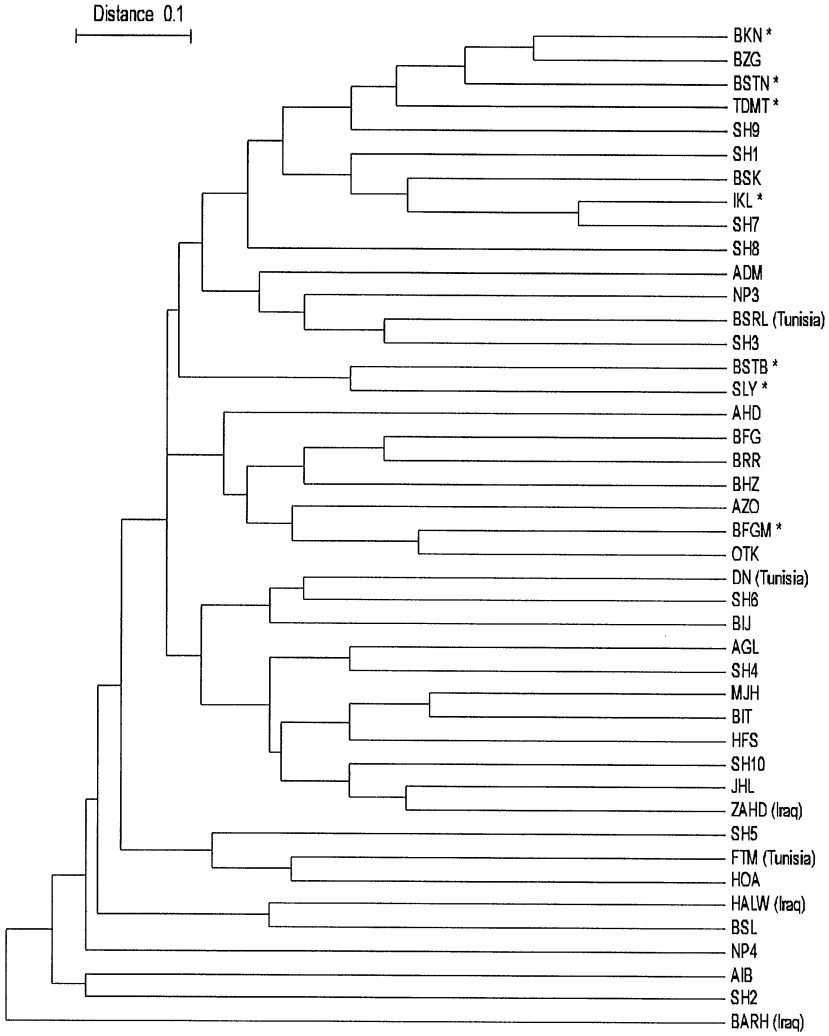

Figure 2. Dendrogram of the accessions listed in Table 1 generated by group average clustering analysis (UPGMA) using RAPD-based genetic distance. The cultivars resistant to 'Bayoud' are indicated by *.

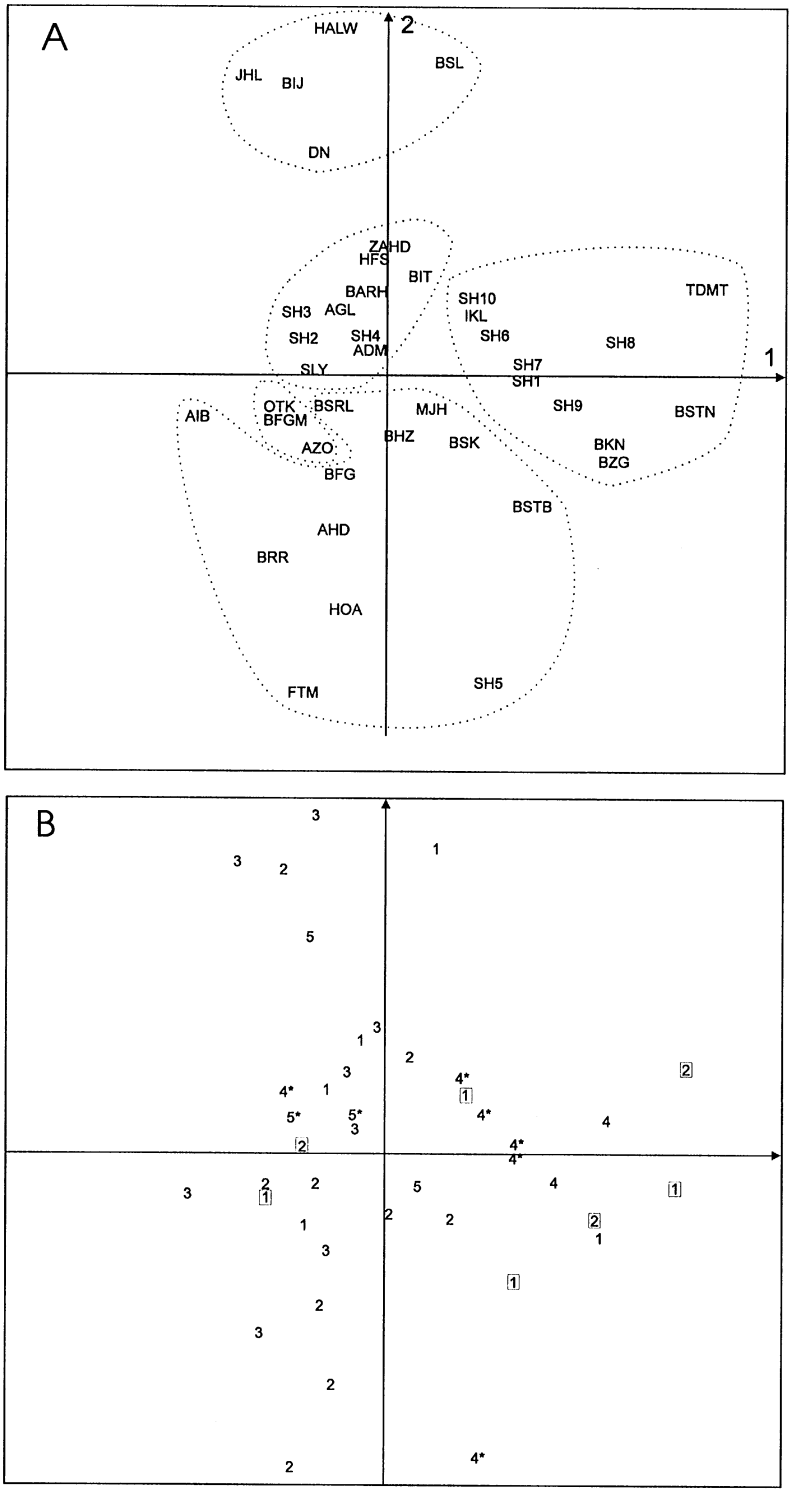

Figure 3. Representations of the 41 female palm accessions on the plane 1–2 of the correspondence analysis. A: The groups deduced from the clustering analysis of factor scores are indicated; B: Each accession is represented by its appearance-value (1 = poor, 2 = medium, 3 = good, 4 = very good, 5 = excellent), the 'Bayoud'-resistant varieties are framed, * indicates the 'khalts' presumed resistant.

with accessions already grown in Morocco. Clustering of the different varieties might possibly be improved by employing additional molecular markers. Nevertheless, the organisation deduced from the RAPD-markers analysis did not appear to be related to date-appearance or resistance to Bayoud (Figure 3). Presence of cultivars showing resistance to Bayoud in different genetic groups may indicate the existence of several genetic resistance sources. If so, combining different sources of resistance could be a valuable breeding strategy.

The relatively low polymorphism and the lack of evident organisation observed among the date palm varieties grown in Morocco could be related to the mode of introduction and maintenance of germplasm. Foundation germplasm is somewhat limited. The fact that the cultivars from Tunisia and Iraq did not markedly diverge from the genetic diversity present in Morocco suggests a narrow genetic diversity of populations from which the present varieties have been derived and maintained over several centuries. Exchange of cultivars between plantations and periodic development of new recombinant cultivars through sexual reproduction and seedling selection may also have played a role. In addition, the selection applied by farmers concerns mainly end-use quality-related genes which may represent only a small fraction of the date palm genome.

In combination with agronomically important morphological criteria, RAPD assay could allow the establishment of a catalogue of cultivars grown world-wide. Other applications could include fingerprinting of date palm genotype, identification of duplicate accessions, and establishment of a core collection.

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References

Aît Chitt, M., S.H. Mantell, M. Thangavelu & C.C. Ainsworth, 1995. Cloning date palm (*Phoenix dactylifera* L.) DNA and characterization of low, medium and high copy DNA sequences. Elaeis (7) 1: 57–63.

Baaziz, M. & M. Saaidi, 1988. Preliminary identification of date palm cultivars by esterase isoenzymes and peroxidase activities. Can J Bot 66: 89–93.

Bendiab, K., M. Baaziz, Z. Brakez & My.H. Sedra, 1993. Correlation of isoenzyme polymorphism and Bayoud-disease resistance in date palm cultivars and progeny. Euphytica 65: 23–32.

Bennaceur, M., C. Lanaud, M.H. Chevalier & N. Bounagua, 1991. Genetic diversity of the date palm (*Phoenix dactylifera* L.) from Algeria revealed by enzyme markers. Plant Breed 107: 56–69.

Benzecri, J.P., 1973. L'analyse des données. Tome 2, L'analyse des correspondances. Dunod, Paris.

Brac de la Perrière, R.A. & A. Benkhalifa, 1991. Progression de la fusariose du palmier dattier en Algérie. Sécheresse 2: 119–128.

Corniquel, B. & L. Mercier, 1994. Date palm (*Phoenix dactylifera* L.) cultivar identification by RFLP and RAPD. Plant Sci 101: 163–172.

Djerbi, M., H. Aouad, H. El Filali, M. Saaidi, A. Chtioui, My.H. Sedra, M. Allaoui, T. Hamdaoui & M. Oubrich, 1986. Preliminary results on selection of high quality Bayoud resistant clones among natural date palm population in Morocco. In: Second Symposium on Date Palm, 3–6 March 1986, King Faisal University, Al-Hassa, Saudi Arabia, pp. 386–399.

Fakir, S., J. Carbonnier & A. Birouk, 1992. Analyse du polymorphisme enzymatique et protéique des cultivars marocains du palmier dattier (*Phoenix dactylifera* L.). In: Complexes d'espèces, flux de gènes et ressources génétiques des plantes. Actes du Colloque International, 8–10/1/92, Paris Lavoisier, pp. 645.

Hu, J. & C.F. Quiros, 1991. Identification of broccoli and cauliflower cultivars with RAPD markers. Plant Cell Rep 10: 505–511.

Koller, B., A. Lehmann, J.M. McDermott & C. Gessler, 1993. Identification of apple cultivars using RAPD markers. Theor Appl Genet 85: 901–904.

Louvet J. & G. Toutain, 1973. Recherches sur les fusarioses. VII. Nouvelles observations sur la fusariose du palmier dattier et précisions concernant la lutte. Ann Phytopathol 5: 35–52.

Lashermes, P., J. Cros, P. Marmey & A. Charrier, 1993. Use of random amplified polymorphic DNA markers to analyse genetic variability and relationships of *Coffea* species. Crop Evol Genet Res 40: 91–99.

Mossler, A., K.N. Egger & G.A. Hughes, 1992. Low levels of genetic diversity in red pine confirmed by random amplified polymorphic DNA markers. Can J For Res 22: 1332–1337.

Pereau-Leroy, P., 1958. Le palmier dattier au Maroc. Ministère de l'Agriculture (142 pp.). Instit Franc Rech Outre-mer, Paris.

Saaidi, M., G. Toutain, H. Bannerot & J. Louvet, 1981. La sélection du palmier dattier (*Phoenix dactylifera* L.) pour la résistance au Bayoud. Fruits 36: 241–249.

Sedra, My.H., 1992. Evaluation and selection of the resistant good cultivars and clones of date palm of the Bayoud disease. Arab Soc Plant Prot 2: 155–160.

Sedra, My.H., 1995. Triage d'une collection de génotypes de palmier dattier pour la résistance au Bayoud causé par *Fusarium oxysporum* f.sp. *albedinis*. Al Awamia 90: 9–18.

Sedra, My.H., H. El Filali & D. Frira, 1993. Observations sur quelques caractéristiques phénotypiques et agronomiques du

fruit des variétés et clones du palmier dattier sélectionnés. Al Awamia 82: 105–120.

Sedra, My.H., H. El Filali, A. Benzine, M. Allaoui, S. Nour & Z. Boussak, 1996. La palmeraie dattière marocaine: Evaluation du patrimoine phénicicole. Fruits 1: 247–259.

Toutain, G., A. Bachra & A. Chari, 1971. Cartographie variétale de la palmeraie marocaine (242 pp.). Direc Rech Agron, Rabat, Morocco.

Van der Peer, Y. & R. De Wachter, 1993. TREECON: a software package for the construction and drawing of evolutionary trees. Comput Applic Biosci 9: 177–182.

Welsh, J. & M. McClelland, 1990. Fingerprinting genomes using PCR with arbitrary primers. Nucl Acid Res 18: 7213–7218.

Williams, J.G.K., A.R. Kubelik, J.L. Kenneth, J.A. Rafalski & S.V. Tingy, 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucl Acid Res 18: 6522–6531.

Wolff, K. & J.P. Van Run, 1993. Rapid detection of genetic variability in chrysanthemum (*Dendranthema grandiflora* Tzvelev) using random primers. Heredity 71: 335–341.

Wrigley, G., 1995. Date palm. In: J. Smartt & N.W. Simonds (Eds.), Evolution of Crop Plants, 2nd ed., pp. 399–403. Longman, London.

Yang, X. & C. Quiros, 1993. Identification and classification of celery cultivars with RAPD markers. Theor Appl Genet 86: 205–212.