Identification of Date Cultivars in California Using AFLP Markers

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Additional index words. genetic similarity and dissimilarity, germplasm, identification, *Phoenix sylvestris*, *Phoenix dactylifera*, polymorphism

Abstract. Polymorphisms of 21 date cultivars (*Phoenix dactylifera* L.) in California were determined by amplified fragment length polymorphism (AFLP) analysis with near infrared fluorescence labeled primers. Four primer sets were used to detect polymorphisms. Based on the UPGMA-cluster analysis of 328 polymorphic bands, the majority of the cultivars was separated into two major groups. Cultivars Abada, Amir Hajj, Ashrasi, Bentamoda, Boyer No. 11, Deglet Beida, Horra, Javis No. 1, Khadrawy, and Thoory belonged to group I. Cultivars Badrayah, Dayri, Halawy, Haziz, Khir, Medjool, Sayer, and Zahidi belonged to group II. Cultivars Barhee and Deglet Noor were further separated from groups I and II. 'Hayany' was distinct from all other date cultivars tested. These results demonstrated that AFLP markers could efficiently identify individual date cultivars. The information will be useful for future date germplasm collection and facilitated selection of diverse parents for cross hybridization in a breeding program.

Dates (*Phoenix dactylifera*) ($2n = 2x = 36$) are dioecious and perennial fruit trees, belonging to the family Arecaceae (Coryphoideae). Thirteen species are now recognized within the genus *Phoenix* (Barrow, 1998). The California date industry was established in the late 19th century using offshoots of many cultivars imported between 1890 to 1922 from different date-growing regions of Algeria, Egypt, Iraq, and Tunisia (Nixon, 1950; Nixon and Carpenter, 1978). Additional cultivars also were introduced into California in recent decades. California produces nearly all the date crops in the United States. The nearly 2,000 ha of date palms in California produce 22,000 tons of dates each year valued between 23–30 million dollars from 1997 to 1999 [U.S. Dept. of Agriculture (USDA) Statistics, 2001]. The United States also has imported between 2,500 to 10,000 tons of dates annually in the past 15 years. The demand for date palm offshoots and mature palms from the landscape industry has been very high for the past few years. The California date industry could expand into this market. In 1999, 892,200 ha of dates were harvested and 5,049,733 tons of dates produced worldwide.

The origin of date palm is unclear because of the long history of cultivation. Date is believed to be a native of western India or the Arabian Gulf region, possibly southern Iraq (Wrigley, 1995). However, genetic relationships among date cultivars remain obscure. Date palms are commonly identified by morphological features, including general mor-

phology, leaf bases, spines, pinnae, fruit stalks, and fruit, that can be used to differentiate 45 imported cultivars in California (Nixon, 1950). However, many of these morphological features, notably fruit features, only occur in mature trees. A minimum of 3–5 years is required before these features can be identified. The morphological features can be affected by environmental factors (i.e., phenotypic plasticity) and may not reflect the true genetic relationships. In recent years, several other methods have been used to identify date cultivars. Morphology of pollen grains was used to identify four male cultivars and other *Phoenix* species (Tisserat and Demason, 1982). In addition, isozyme markers estimated the genetic variability among date cultivars, although not all cultivars tested could be differentiated (Al-Jibouri and Adham, 1990; Baaziz and Saaidi, 1988; Bennaceur et al., 1991; Torres and Tisserat, 1980). RFLP analysis with a single cDNA probe differentiated four date cultivars: 'Kenessy', 'Lulu', 'Nabtha Saïf', and 'Sheshi'. (Corniquel and Mercier, 1997). RFLP and RAPD markers identified five date cultivars: 'Barhee', 'Deglet Noor', 'Khadrawy', 'Khalassa', and 'Medjool' (Corniquel and Mercier, 1994). RAPD markers also identified date palm accessions in Egypt, Morocco, and Tunisia (Mokhtar et al., 1998; Saker and Moursy, 1998; Sedra et al., 1998).

Amplified fragment length polymorphisms (AFLP) are polymerase chain reaction (PCR) based markers (Vos et al., 1995) that have been reliably used for genome mapping and examination of genetic diversity between closely related species, populations, or both. AFLP markers have evaluated diversity and relationships in many fruit crops, such as apple (*Malus domestica* Borkh.) (Goulão et al., 2001), banana (*Musa* L.) (Loh et al., 2000), coconut (*Cocos nucifera* L.) (Perera et al., 1998), grape (*Vitis vinifera* L.) (Cervera et al., 1998), olive (*Olea europaea* L.) (Angiolillo et al., 1999),

peach (*Prunus persica* L.) (Dirlewanger et al., 1998), pear (*Pyrus communis* L.) (Monte-Corvo et al., 2000), and sweet cherry (*Prunus avium* L.) (Struss et al., 2001). AFLP markers also were used in date palm breeding program for potential mapping population (El-Kharbotly et al., 1998). Thirty-five primer sets were used to detect polymorphic bands between parental cultivars Um-Assla and KL-96. On average, 25.7 bands were detected per primer set and with 9.4 polymorphic bands per primer set. The percentage of polymorphic bands ranged from 4% to 55% depending on the primer combination. The objectives of this study were to develop a date palm cultivar identification system in California based on AFLP markers to confirm the identity of young date palm offshoots and to determine the genetic relationship among diverse California-grown date palm cultivars.

Materials and Methods

Plant materials. Young leaves of 21 date cultivars were collected from the USDA germplasm collection at Coachella Valley Agricultural Research Station (CVARS), Univ. of California-Riverside, Thermal, Calif. (Table 1). The sample of cultivar Dayri was obtained from commercial planting in Coachella Valley. *A. P. sylvestris* (also called wild date or Indian date) individual from the germplasm collection was included in the study for comparison. This species is a species very closely related to *P. dactylifera* (Barrow, 1998).

DNA extraction and AFLP reaction. Total DNA was isolated from young leaves using a C-TAB extraction method (Doyle and Doyle, 1987) and quantified by Hoefer DyNA Quant200 (Pharmacia Biotech, Piscataway, N.J.). AFLP analysis was conducted using the GIBRL BRL AFLP System II (Life Technologies, Grand Island, N.Y.) and visualized with the LI-COR IR automated sequencer 4000L (LI-COR, Lincoln, Nebr.). Genomic DNA (125 ng) was digested with 1 µL of mixture of *EcoR* I/*Mse* I (1.25 units/µL) at 37 °C overnight, and ligated to *EcoR* I/*Mse* I adapters with 1.5 µL (1 unit/µL) of T4 DNA ligase at 25 °C for at least 6 h. For the pre-amplification reaction, we used a mixture of 2.5 µL of DNA from the ligation reaction, 20 µL of Pre-amp mix II, 2.5 µL of 10× PCR buffer, and 0.2 µL of *Taq* DNA polymerase (5 units/µL). The pre-amplification reactions were performed on a MJR Cycle LR thermocycler (MJ Research, Watertown, Mass.) with a profile as follows: 30 cycles at 94 °C for 15 s, 56 °C for 30 s + 1 s/cycle, and 72 °C for 1 min + 1 s/cycle, then 1 cycle at 72 °C for 3 min. For the selective amplification, we incorporate the near infrared fluorescence dye (IRD) labeled *EcoR* I primers with the pre-amplification product: 2 µL of DNA from pre-amp, 2 µL of *Mse* I primer, 0.5 µL of IRD700-labeled *EcoR* I primer, 0.5 µL of IRD800-labeled *EcoR* I primer, 1 µL of 10× PCR buffer, 4 µL of H₂O, and 0.16 µL of *Taq* DNA polymerase (5 units/µL). The selective amplification PCRs were performed by a touchdown program as follow: 13 cycles at 94 °C for 15 s, 65 °C for 30 s – 0.7 °C/cycle, and 72 °C

Received for publication 20 July 2001. Accepted for publication 10 Feb. 2002. We acknowledge R. Krueger, and V. Samons for the assistance in the collection of date palm cultivar samples. We also recognize partial financial support from the Univ. of California, Genetic Resources Conservation program.

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for 1 min, then 30 cycles at 94 °C for 15 s, 56 °C for 30 s + 1 s/cycle, 72 °C for 1 min + 1 s/cycle, then 72 °C for 3 min. Both pre- and selective-amplification conditions were modified according to Myburg et al. (2000). In order to identify the primer combinations revealing clear, reproducible polymorphisms, we screened 32 primer combinations using two date cultivars (Deglet Noor and Medjool). The products from the selective amplification were electrophoresed on a 25-cm × 0.25-mm 8% denaturing polyacrylamide long ranger gel solution (BMA, Rockland, Maine) with 0.8× TBE buffer using a LI-COR automated sequencer 4000L. The gel was pre-run for 10 to 20 min at 1500 V, 40 mA, and 40 W until the gel temperature reached 50 °C. The samples were denatured at 95 °C for 3 min and immediately placed on ice. We loaded 1.15 µL of samples and 1 µL of mixture of IRD700 and IRD800 size markers. Samples were electrophoresed at 1500 V, 50 °C for 3.5 h.

Data analysis. All AFLP analyses were performed in duplicates. Clear and unambiguous AFLP bands were scored as present (1) or absent (0) using the Cross Checker software (version 2.9) (<http://www.spg.wau.nl/pv/pub/CrossCheck>) to create the binary data set. The bands were re-checked visually to avoid any misinterpretation of the scored bands. A dendrogram based on genetic distance was constructed by NTSYS version 2.1 (Exeter Software, Setauket, N.Y.) (Rohlf, 2000) based on the unweighted pair group method with arithmetic average (UPGMA).

Results and Discussion

AFLP fingerprinting and polymorphism in date cultivars. Initially, 32 primer combinations were screened using two date cultivars (data not shown). Generally, primer pairs produced 50–70 bands, with band size ranging from 50–700 bp. The number of AFLP bands that we detected using the fluorescence labeled AFLP method is much higher than the average 25.7 bands tested in date palm using the radioactive (³²P) labeled AFLP method (El-Kharbotly et al., 1998). Direct comparison between the two labeling method showed that the fluorescence labeled AFLP method offered significant improvement of fragment sizing and data handling (Zhang et al., 1999). It was also more accurate and more efficient. Based on the initial screening, four primer combinations, IRD800 E-AC/M-CAG, IRD700 E-TA/M-CAG, IRD800 E-AG/M-CAT, and IRD700 E-TG/M-CAT, were selected for the study of date cultivars. These four primer sets generated clear, consistent patterns for 21 date cultivars and *P. sylvestris*. A total of 328 polymorphic AFLP bands were scored for all the samples. On average, each primer combination generated ≈70 polymorphic bands. An example of the AFLP fingerprinting with primer set IRD800 E-AG/M-CAT is shown (Fig. 1) where majority of the 21 date cultivars and *P. sylvestris* were uniquely identified.

Genetic diversity among date cultivars. UPGMA analysis of 21 date cultivars and *P.*

Table 1. Date cultivars used in the study, their plant introduction (P.I.) number, accession number. (ACC No.), gender, and countries of introduction or selection.

Cultivars	P.I. no.	ACC no.	Gender	County of introduction or selection
Abada	555400	78-13	F	California
Amir Hajj	80781	78-12	F	Iraq
Ashrasi	8739	78-14	F	Iraq
Badrayah	80789	78-18	F	Iraq
Barhee	8746	78-15	F	Iraq
Bentamoda	36818	78-19	F	Sudan
Boyer no.11	555420	78-8	M	California
Dayri			F	Iraq
Deglet Beida	10834	78-16	F	Algeria
Deglet Noor	4611	78-22	F	Algeria
Halawy	8750	78-23	F	Iraq
Hayany		96-01	F	Egypt
Haziz	555442	78-24	F	California
Horra	15026	78-17	F	Tunisia
Javis no.1	555443	78-11	M	California
Khadrawy	8751	78-21	F	Iraq
Khir	11801	78-28	F	Saudi Arabia
Medjool	74204	78-43	F	Morocco
Sayer	8748	78-31	F	Iraq
Thoory	10891	79-16	F	Algeria
Zahidi	8743	79-17	F	Iraq
<i>P. sylvestris</i>		90-1	F	Unknown

Fig. 1. AFLP patterns of 21 date palm cultivars and *P. sylvestris*. The amplified bands ranging from 50 bp to 500 bp using the primer set IRD800 E-AG/M-CAT. Samples from left to right are: (1) Bentamoda; (2) Halawy; (3) Badrayah; (4) Ashrasi; (5) Amir Hajj; (6) Abada; (7) Boyer No. 11; (8) Barhee; (9) Deglet Noor; (10) Deglet Beida; (11) Khadrawy; (12) Horra; (13) Haziz; (14) Jarvis No. 1; (15) Medjool; (16) Khir; (17) Zahidi; (18) Dayri; (19) Sayer; (20) Thoory; (21) Hayany; (22) *P. sylvestris*; (M) LI-COR 50–700 bp IRD-labeled markers (LI-COR, Lincoln, Nebr).

Fig. 2. Dendrogram derived from a UPGMA analysis using the Jaccard's coefficient based on 328 polymorphic AFLP bands showing the relationship among 21 date palm cultivars and *P. sylvestris*.

sylvestris based on AFLPs differentiated two major groups based on AFLP markers (Fig. 2). Both groups included cultivars originally introduced from Iraq or Algeria. Dates have been cultivated in the regions of North Africa, the Mediterranean, and Arabian Peninsula for over 5000 years (Wrigley, 1995; Zohary and Hopf, 1988). Complex introductions and exchanges of germplasm between date growing countries occurred through out the long history. Thus, the countries from which these date cultivars were introduced into the United States, may not reflect their origin. The two groups of date cultivars identified in the study may represent two groups of dates from different geographic origins or could represent groups separated by biological barriers. Regardless of the basis for their distinct genetic patterns, these two groups should be intercrossed in future breeding programs. Cultivars Barhee and Deglet Noor are more distant from both groups and 'Hayany' formed a fourth group. *P. sylvestris* was positioned outside of the date cultivars, but was similar enough to dates to suggest that interspecific hybrids between *P. dactylifera* and *P. sylvestris* may have occurred. Other types of molecular markers such as micro-satellite markers and comparisons of genetic maps will be informative in determining the possibility of hybrid origins. A larger and more diverse collection of date cultivars in all date growing countries should also be undertaken.

Sedra et al. (1998) found low levels of polymorphism using the RAPD markers among date accessions in Morocco. In contrast, our AFLP marker system demonstrated polymorphisms that differentiated each of the date cultivars. AFLP fingerprinting is recommended for future date cultivar identification, identification of duplicated and mixed accessions, determination of genetic relationships among germplasm introductions, evaluation of the genetic identity and changes in tissue culture

derived date palms, confirmation of the identity of off-shoots during propagation, and study of genetic strains within the same cultivar.

Literature Cited

- Al-Jibouri, A.A.M. and K.M. Adham. 1990. Biochemical classification of date palm male cultivars. *J. Hort. Sci.* 65:725-729.
- Angiolillo, A., M. Mencuccini, and L. Baldoni. 1999. Olive genetic diversity assessed using amplified fragment length polymorphisms. *Theor. Appl. Genet.* 98:411-421.
- Baaziz, M. and M. Saaidi. 1988. Preliminary identification of date palm cultivars by esterase isoenzymes and peroxidase activities. *Can. J. Bot.* 66:89-93.
- Barrow, S. 1998. A monograph of *Phoenix* L. (Palmae: Coryphoideae). *Kew Bul.* 53:513-575.
- Bennaceur, M., C. Lanaud, M.H. Chevallier, and N. Bounaga. 1991. Genetic diversity of the date palm (*Phoenix dactylifera* L.) from Algeria revealed by enzyme markers. *Plant Breeding* 107:56-69.
- Cervera, M.T., J.A. Cabezas, J.C. Sancha, F. Martinez de Toda, and J.M. Martinez-Zapater. 1998. Application of AFLPs to the characterization of grapevine *Vitis vinifera* L. genetic resources. A case study with accessions from Rioja (Spain). *Theor. Appl. Genet.* 97:51-59.
- Corniquel, B. and L. Mercier. 1994. Date palm (*Phoenix dactylifera* L.) cultivar identification by RFLP and RAPD. *Plant Sci.* 101:163-172.
- Corniquel, B. and L. Mercier. 1997. Identification of date palm (*Phoenix dactylifera* L.) cultivars by RFLP: Partial characterization of a cDNA probe that contains a sequence encoding a zinc finger motif. *Intl. J. Plant Sci.* 158:152-156.
- Dirlwanger, E., V. Pronier, C. Parvery, C. Rothan, A. Guye, and R. Monet. 1998. Genetic linkage map of peach [*Prunus persica* (L.) Batch] using morphological and molecular markers. *Theor. Appl. Genet.* 97:888-895.
- Doyle, J.J. and J.L. Doyle. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochem. Bul.* 19:11-15.
- El-Kharbotly, A., M.E. Mardi, N.A. Saadi, and Y. Al-Mahruki. 1998. Proc. The First Intl. Conf. on

- Date Palms. Al-Ain, United Arab Emirates, p. 194-207.
- Goulão, L., L. Cabrita, C.M. Oliveira, and J.M. Leitão. 2001. Comparing RAPD and AFLP analysis in discrimination and estimation of genetic similarities among apple (*Malus domestica* Borkh.) cultivars. *Euphytica* 119:259-270.
- Loh, J.P., R. Kiew, O. Set, L.H. Gan, and Y-Y. Gan. 2000. Amplified fragment length polymorphism fingerprinting of 16 banana cultivars (*Musa* cvs.). *Mol. Phylogenet. Evol.* 17:360-366.
- Mokhtar, T., B. Abdel Ali, R. Abdelmajid, R. André, and M. Mohamed. 1998. Molecular characterization of Tunisian date palm varieties. *Proc. The First Intl. Conf. on Date Palms*. Al-Ain, United Arab Emirates, p. 183-193.
- Monte-Corvo, L., L. Cabrita, C. Oliveira, and J. Leitao. 2000. Assessment of genetic relationships among *Pyrus* species and cultivars using AFLP and RAPD markers. *Genet. Resources Crop Evol.* 47:257-265.
- Myburg, A.A., D. O'Malley, R.R. Sederoff, and R. Whetten. 2000. High-throughput multiplexed AFLP analysis of interspecific hybrids of *Eucalyptus* trees species. *Plant & Animal Genome VIII Conf.*, San Diego, p. 544.
- Nixon, R.W. 1950. Imported cultivars of dates in the United States. *USDA Circular No.834*. p.144.
- Nixon, R.W. and J.B. Carpenter. 1978. Growing dates in the United States. *USDA Bul. No. 207*.
- Perera, L., J.R. Russell, J. Provan, J.W. McNicol, and W. Powell. 1998. Evaluating genetic relationship between indigenous coconut (*Cocos nucifera* L.) accessions from Sri Lanka by means of AFLP profiling. *Theor. Appl. Genet.* 96:545-550.
- Rohlf, F.J. 2000. NTSYSpc numerical taxonomy and multivariate analysis system, version 2.1 user guide. Exeter Software, Setauket, N.Y.
- Saker, M.M. and H.A. Moursy. 1998. Molecular characterization of Egyptian date palm: II. RAPD fingerprints. *Proc. The First International Conference on Date Palms*. Al-Ain, United Arab Emirates, p. 173-182.
- Sedra, M.H., P. Lashermes, P. Trouslot, M. Combes, and S. Hamon. 1998. Identification and genetic diversity analysis of date palm (*Phoenix dactylifera* L.) cultivars from Morocco using RAPD markers. *Euphytica* 103:75-82.
- Struss, D., M. Boritzki, K. Glozer, and S.M. Southwick. 2001. Detection of genetic diversity among population of sweet cherry (*Prunus avium* L.) by AFLPs. *J. of Horticultural Science & Biotechnology* 76:362-367.
- Tisserat, B. and D.A. DeManson. 1982. A scanning electron microscope study of pollen of *Phoenix* (Arecaceae). *J. Amer. Soc. Hort. Sci.* 107:883-887.
- Torres, A.M. and B. Tisserat. 1980. Leaf isozyme as genetic markers in date palms. *Amer. J. Bot.* 67:162-167.
- U.S. Department of Agriculture Statistics, 2001. <http://www.nass.usda.gov/census>
- Vos, P.R., M. Hogers, M. Bleeker, T. van de Lee Reijans, M. Hornes, A. Fritjers, J. Pot, J. Peleman, M. Kuiper, and M. Zabeau. 1995. AFLP: New concept for DNA fingerprinting. *Nucleic Acids Res.* 23:4407-4414.
- Wrigley, G. 1995. Date palm, p. 399-403. In: J. Smartt and N.W. Simmonds (eds.). *Evolution of crop plants*, second edition. Longman Group, Essex, England.
- Zohary, D. and M. Hopf. 1988. Domestication of plants in the Old World: The origin and spread of cultivated plants in West Asia, Europe and the Nile Valley. Clarendon Press, Oxford.