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BRIEF COMMUNICATION

Development and evaluation of microsatellite markers in *Phoenix dactylifera* L. and their transferability to other *Phoenix* species

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Abstract

Forty one simple sequence repeats were isolated from two microsatellite enriched libraries of date palm (*Phoenix dactylifera* L.). After screening, 17 selected microsatellite loci were characterized and evaluated on a set of 31 cultivars and clones from Algerian and Californian germplasm. All primer pairs produced an amplification product of the expected size and detected high polymorphism among the analysed samples. These nuclear simple sequence repeat (SSR) markers are expected to be a very effective tool for evaluating genetic diversity in date palm germplasm. Across-taxa amplification showed the usefulness of most SSR markers in 14 other species across the genus *Phoenix*.

Additional key words: across-taxa transferability, date palm, simple sequence repeats.

Date palm (*Phoenix dactylifera* L.) is a long-lived dioecious monocotyledonous fruit plant (2n=36) belonging to the *Arecaceae* family (Barrow 1998). It is believed to have originated in Mesopotamia and thousands of cultivars have been reported (Hanachi *et al*. 1998). Date palms have always been clonally propagated to ensure the identity and uniformity of the cultivars. However, the existence of intra-cultivar variation could potentially cause confusion in cultivar nomenclature, preservation and utilization (Devanand and Chao 2003).

Discrimination among closely related cultivars and clones is often extremely difficult. Identification of date palm cultivars is principally based on fruit morphology (Elhoumaizi *et al*. 2002). However, morphological traits are often unreliable or imprecise indicators of plant genotype, being influenced by environmental conditions or varying with the developmental stage of plants. DNA typing can be a convenient method for accurately identifying date palm cultivars and analysing their genetic diversity and phylogenic relationships. Microsatellite or simple sequence repeat (SSR) molecular markers have been proven to be very powerful in plant diversity analysis because they are locus-specific, codominant, highly polymorphic and highly reproducible. Recently, microsatellite markers have been developed and used to investigate genetic diversity in *P. dactylifera* (Billotte *et al.* 2004). However, for a wider use of this technology, the enlargement of the number of markers suitable for evaluating DNA polymorphisms in this crop is necessary.

In this study, we report the development of 17 new SSR markers for *P. dactylifera*. Their polymorphism was studied in a set of 31 cultivars and clones of this species. Their cross-species transferability was evaluated on 14 other *Phoenix* species. Such investigation would be of great interest in breeding programs (Ould Mohamed Salem *et al.* 2007).

Leaf samples of *P. dactylifera* were collected from 31 cultivars and clones from three Algerian oasis (Adrar, Biskra, Golea) and from the USDA-ARS National Germplasm Repository (California). Total genomic DNA

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Abbreviation: SSRs - simple sequence repeats.
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was isolated from the freeze-dried leaf tissue according to Thomas *et al.* (1993) with modifications. In addition, DNA samples from 14 *Phoenix* species such as *P. acaulis* Roxb., *P. canariensis* Chabaud, *P. loureiri* Kunth, *P. paludosa* Roxb., *P. reclinata* Jacq., *P. rupicola* T. Anderson, *P. sylvestris* (L.) Roxb., *P. theophrasti* Greuter, *P. caespitosa* Chiov., *P. farinifera* Roxb., *P. hanceana var. formosa* Becc., *P. reclinata* Jacq. *P. roebelenii* O'Brien, *P. zeylanica* Trimen, were provided by the CIRAD (France) and the Royal Botanic Gardens, Kew (UK).

Genomic DNA from *P. dactylifera* cv. Deglet Nour was digested with *Rsa*I restriction enzyme and ligated with 21 mer and 25 mer Mlu adapter (Edwards *et al.*

Table 1. Microsatellite loci used for genotyping in date palm cultivars. Repeat motifs, GenBank accessions, primer sequences, allele size ranges in *P dactylifera* cultivars and other *Phoenix* species (in parentheses), number of alleles (Na), expected heterozygosity (He), observed heterozygosity (Ho), frequency of null alleles (r), polymorphism information content (PIC) of 17 SSR loci analysed in 31 *P. dactylifera* cultivars and clones. Loci in Hardy-Weinberg equilibrium are indicated by *. Annealing temperature was 55 °C.

Locus	Repeat motif	Accession	Primer sequences (5' - 3')	Allele size [bp]	Na	He	Ho	r	PIC
PDCAT1*	$(TC)_{21}$	EF015858	CTGAAATCTCTGTTCAAATCC AGTTTGGATCTATTTGTGAGT TATTTTCTTT	76-134 (71-130)	8	0.73	0.37	0.206	0.70
PDCAT2*	$CTCGCTG(TC)_3(TC)_3T$ $(TC)_3T(TC)_3T(TC)_4TTCT$ $GTCCCG(TC)_{16}T(TC)$	EF015859	GGCCTTCTCTTCCCTAATGGG AGTTTCTTGCCCCTGTTCTTTC CCTC	166-194 (148-197)	8	0.81	0.96	-0.081	0.79
PDCAT3	$(CA)_8$ - $(GT)_3(CA)_4$	EF015860	CAAGGATAGGTGTGATGACC ACCGTTTGTCCTTTTAACTTCT TGCTGGAATT	225-229 (227-239)	3	0.47	0.42	0.035	0.39
PDCAT4	$(CA)_8TT(CA)_4(GA)_{20}$	EF015862	TAACGAGTCCACACACCTGGG TAAAGCTTATAAG	124-170 (103-250)	8	0.65	0.58	0.042	0.62
PDCAT5*	$(AG)_{16}$	EF015863	GGCCCGTCCTTGGATTAGAGC TACGTTGTCCCGTCAATTGG	66-90 (65-119)	4	0.61	0.42	0.120	0.55
PDCAT6	$(CA)_{14}(GA)_{23}$	EF015864	AATCAGGGAAACCACAGCCA GTTTAAAGCCTTCTCAAGATA GCCTCAG	117-165 (82-188)	9	0.85	0.83	0.008	0.83
PDCAT8*	$(TC)_{16}$	EF015866	GCTTAAGTGGTTAGTTGCCAA GTTTGGCAGAAGTATTGAAA AGTTGA	201-237 (110-135)	9	0.80	0.83	-0.016	0.78
PDCAT10	$(TC)_{16}$	EF015868	CACTGCTCCTGTTGCCCTGTT GTAGAAGGGCAGAGGACGG	110-112 (107-127)	2	0.25	0.29	-0.034	0.22
PDCAT11*	$(TC)_7(TC)_{20}$	EF015869	TTAGTAGACTCCCCACCGTCC TGTTTCATGGTGCTGGAGAAT GAA	133-155 (113-145)	8	0.79	0.92	-0.072	0.76
PDCAT12*	$(CT)_{19}$	EF015870	CATCGTTGATTCCTAACCCCT CGTTTAGATCTTGCATGGCAA CGC	145-167 (140-180)	4	0.72	0.04	0.395	0.67
PDCAT13*	$(GA)_{21}GCA(GGA)GA$ $(GGA)_3$	EFO15871	TGTTGCCATTCACATGCTGCG TTTGGACTAGTCCCTCCCTCCC	133-173 (128-211)	8	0.70	0.54	0.095	0.68
PDCAT14	$(TC)_{19}(TC)_{16}$	EF015872	TGCTGCAAATCTAGGTCACGAG TTTACCCCTCGGCCAAATGTAA	114-145 (101-155)	8	0.81	0.92	-0.058	0.79
PDCAT15	$(GA)_{13}$-$(GA)_8(GA)_6$	EF015873	ACAGAGAGGTGGAGTTTTCGG ATTTCTTCCTTTCAAACCAGCA AGCT	128-134 (127-163)	3	0.16	0.17	-0.010	0.15
PDCAT17	$(GA)_{21}$	EF015875	CAGCGGAGGGTGGGCCTCGTT TCTCCATCTCCCTTTTTCTTCT GCTACTC	116-145	7	0.77	0.71	0.036	0.74
PDCAT18	$(CT)_{13}G(CT)_8CG(CT)_3$ $CG(CT)_3$	EF015876	CCTAAACCTGAATGAATCAAAG CAACTAACATAAGGACAGTGCT ATGTGATTG	111-150	8	0.72	0.79	-0.041	0.68
PDCAT20*	$(GA)_{29}$	EF015878	TTTCAGACACATCAAGTAACG ATGAGTTTACGTCCACCCCAA GTTACGA	294-353	9	0.79	0.58	0.114	0.75
PDCAT21*	$(GA)_5T(GA)_2TA(GA)_2$ $GC(GA)_5(GT)_7$	EF015861	GTGTTTGAAGATTGATTTTGT GTTATGAGGTTTCGAACTATA GGCATGCACAATAGTATATTG	144-150	3	0.62	0.50	0.074	0.54

1996). GA and GT repeats were enriched using GA and GT oligo, *Streptavidin*-coated magnetic beads (*Dynel*, Oslo, Norway). The microsatellite-enriched DNA was amplified using 21 mer primer-adapter, purified and cloned into BssII site of pBluscript II SK (+) (*Stratagen*, La Jolla, USA), which was later transformed into DH5α competent cells (*Invitrogen*, Carlsbad, USA).

Forty one SSR-containing DNA fragments were obtained out of 50 clones sequenced at random using the Bigdye 1.1 kit (*Applied Biosystems*, Foster City, USA) with M13 forward primer, and *Abi-Prism 377* sequencer (*Applied Biosystems*). All sequences obtained were blasted each other using the *Bio-edit v.5.0.9* package (http://www.mbio.ncsu.edu/bioedit/bioedit.html) software and redundant sequences were removed. At first, a set of 21 primer pairs for microsatellite amplifications was designed using the software *Primer Express 1.0* (*Applied Biosystems*). The polymerase chain reaction (PCR) amplification was performed on 50 ng DNA in a 0.02 cm^3 final volume containing 0.5 unit of *AmpliTaq Gold* polymerase (*Applied Biosystems*), 0.002 cm^3 10× PCR buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl), 1.5 mM $MgCl_2$, 200 μM dNTPs and 0.5 μM of each primer. The PCR programme was an initial denaturation of 9 min at 95 °C, 28 cycles of 30 s at 95 °C, 45 s at 55 °C, and 1 min at 72 °C with a final elongation step of 45 min at 72 °C. Forward primers were labelled with a fluorochrome (6-FAM, HEX, NED and PET) and PCR products were resolved on an *Abi-Prism 377* sequencer. Data were analysed using the software *Identity 1.0* (Wagner and Sefc 1999) and *Power marker version 3.02* (http://www.powermarker.net). Loci were evaluated for Hardy-Weinberg equilibrium using *Popgene 1.32* software (Yeh and Boyle 1997).

In a small progeny of five seedlings, alleles at all loci appeared to segregate according to Mendelian expectations. All SSR primer pairs amplified successfully in all 31 *P. dactylifera* accessions analysed except one locus. Nine loci are in Hardy-Weinberg equilibrium ($P \leq 0.05$), while eleven are not in equilibrium and this, probably, can be due to the high inbreeding of the cultivars analysed. Observed (Ho) and expected (He) heterozygosity, frequency of null alleles (r), and polymorphism information content (PIC) were nevertheless calculated in all loci to evaluate their information (Table 1). The polymorphism and discriminant power of each locus (Table 1) were evaluated on the basis of the number of alleles (mean 6.4), He (mean 0.66), and PIC (mean 0.63). Further three microsatellites loci were removed due their monomorphism. PDCAT1, PDCAT2, PDCAT6, PDCAT8, PDCAT11, PDCAT13, PDCAT14, PDCAT17, PDCAT18, and PDCAT20 are the most interesting loci for fingerprinting on the basis of their high level of polymorphism. Moreover, PDCAT4 can be considered promising; their PD and He values are above average and thus a high level of polymorphism is expected once a larger number of genotypes is analysed. Less polymorphic loci (PDCAT3, PDCAT5, PDCAT10, PDCAT12, PDCAT15, and PDCAT21) might be useful for genetic mapping and other purposes. The transferability of microsatellite loci was evaluated in further 14 *Phoenix* species. All tested primer pairs amplified DNA fragments in all species accessions, confirming the conservation of the priming sites flanking the microsatellite loci (Table 1). Furthermore, cross-species amplification revealed clear SSR pattern and polymorphism within the expected allelic range, except for the locus PDCAT4, possibly due to base mutational event in the annealing site.

The newly isolated microsatellite markers are expected to provide a valuable and highly informative resource for diversity analysis in date palm and in genetic mapping.

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