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Primer Note

In silico mining of microsatellites in coding sequences of the date palm (Arecaceae) genome, characterization, and transferability[1]

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- *Premise of the study:* To complement existing sets of primarily dinucleotide microsatellite loci from noncoding sequences of date palm, we developed primers for tri- and hexanucleotide microsatellite loci identified within genes. Due to their conserved genomic locations, the primers should be useful in other palm taxa, and their utility was tested in seven other *Phoenix* species and in *Chamaerops*, *Livistona*, and *Hyphaene*.
- *Methods and Results:* Tandem repeat motifs of 3–6 bp were searched using a simple sequence repeat (SSR)–pipeline package in coding portions of the date palm draft genome sequence. Fifteen loci produced highly consistent amplification, intraspecific polymorphisms, and stepwise mutation patterns.
- *Conclusions:* These microsatellite loci showed sufficient levels of variability and transferability to make them useful for population genetic, selection signature, and interspecific gene flow studies in *Phoenix* and other Coryphoideae genera.

Key words: Arecaceae; Coryphoideae; microsatellite/SSR mining; *Phoenix dactylifera*; transferability.

The date palm (*Phoenix dactylifera* L.) is a monocotyledon species belonging to the Arecaceae family, and is widely cultivated in North Africa, the Sahel (from the Atlantic to the Red Sea), the Middle East, and eastward to the Indus Valley. The date palm is well adapted to cultivation in arid and semiarid areas, and it has been introduced in warm and dry regions worldwide. Mainly grown for its fruits, the date palm represents an important ecological and socioeconomic resource.

Despite the increasing number of studies on date palm, there are still not enough molecular markers available for a number of applications. Most published microsatellite or simple sequence repeat (SSR) markers are dinucleotide loci from unknown noncoding regions of the genome, generally isolated from microsatellite-enriched DNA libraries (Billotte et al., 2004; Arabnezhad et al., 2012). The increasing amount of available genome sequence data offers new prospects for microsatellite marker development through in silico mining, a promising approach for date palm (Cherif et al., 2013), based on the recently published date palm genome sequence (Al-Dous et al., 2011) and expressed sequence tags (ESTs) (Zhao et al., 2012). Our aim was to develop new markers from coding sequences to ensure clear stepwise mutation patterns usable for genetic diversity, dating, and selection signature analyses, and also to facilitate transferability to other species.

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METHODS AND RESULTS

In silico microsatellite mining and primer design were performed on the date palm genome draft sequence version 2 (Al-Dous et al., 2011), with the Perl script SSR_pipeline-v2.pl (Poncet et al., 2006), which incorporates three free software programs: Tandem Repeats Finder (Benson, 1999), Primer3 (Rozen and Skaletsky, 2000), and BLAST (Altschul et al., 1990). The multi-FASTA file of all 19,414 predicted genes (full and partial; PDK20.mRNA.fsa) and the multi-FASTA file with all scaffold sequences (PDK20.fsa) from version 2 of the date palm genome research program at Weill Cornell Medical College in Qatar were downloaded from http://qatar-weill.cornell.edu/research/datepalmGenome/download.html. The search identified 204 genes containing coding sequences with microsatellites, 150 of which were suitable for primer design, but only 103 had nonduplicated primer annealing sites. Among them, we retained loci having perfect trinucleotide motifs with six (excluding those without annotation) or more (with or without annotations) repeats, and hexanucleotide motifs with at least four repeats (with or without annotation).

Of the 47 primer pairs finally retained, 33 generated expected PCR amplification patterns in a preliminary test with eight *P. dactylifera* individuals (Table 1). The 33 loci were further tested on 16 individuals representing *P. dactylifera* (7),

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TABLE 1. Characteristics of 33 microsatellite markers developed for *Phoenix* species. The putative annotation was done using the BLASTX program and the UniProtKB/Swiss-Prot protein database with an *E*-value cutoff of 10^{-5}.

Locus	Primer sequences (5′–3′)[a]	Repeat motif	Size range (bp)[b]	Scaffold ID	Start	Stop	Gene annotation	*E*-value	Organism
mPdIRD01	F: CTCGGAAGGGTATGGACAAA R: TTGCCTTCGACGTGGTAGTA	$(AAG)_3$	200	PDK_20s1306691	24393	24401	Putative pectinesterase/pectinesterase inhibitor 28	4.00E-87	*Arabidopsis thaliana*
mPdIRD03	F: CATTGATCCAACACCACCAC R: GCCAAAACCAGCTCTGGTAAC	$(CCT)_6$	192–198	PDK_20s1315791	3431	3448	Cysteine-rich receptor-like protein kinase 2	1.00E-166	*Arabidopsis thaliana*
mPdIRD04	F: TTGGTGGCCTTTCTCAGAGT R: TGGGATCAAAGTAGGGTTGG	$(AGC)_6$	255–261	PDK_20s13282911	9405	9422	*S*-adenosylmethionine synthase 10	6.00E-77	*Oryza sativa*
mPdIRD05	F: CTATCAGGATGGGGGTGATG R: ACCCATCTGCATAGCTCCAG	$(GAT)_6$	301–302	PDK_20s1366071	11666	11683	DEAD-box ATP-dependent RNA helicase ISE2, chloroplastic	3.00E-09	*Arabidopsis thaliana*
mPdIRD07	F: TGCAATACGATGGCAGAGTC R: CCTTGCAAGTTTTCCACACC	$(TGG)_6$	182–212	PDK_20s1387131	3737	3754	No hit	–	–
mPdIRD08	F: CTATTGGGTCCCTTGGTGAG R: TGACTGCTCGTCATCAGGTC	$(GAT)_6$	202	PDK_20s1402051	10945	10962	No hit	–	–
mPdIRD10	F: ATGCGTTCATCTCCCTTGAG R: GCTGCAAACATCATCCTCAC	$(CAG)_6$	194–214	PDK_20s1405881	31976	31993	No hit	–	–
mPdIRD11	F: GAGTTGGAGGCAAAACCAGA R: CCACAAAACCCTTGTCTTCC	$(GAT)_6$	309–317	PDK_20s1422271	4385	4402	Two-component response regulator-like APRR9	5.00E-18	*Arabidopsis thaliana*
mPdIRD13	F: GCGGAGACAGGAGATGGTAA R: CTTGACTGCTTCTGCTGCTG	$(CAC)_6$	198–227	PDK_20s1496731	12538	12555	Trihelix transcription factor GT-2	8.00E-62	*Arabidopsis thaliana*
mPdIRD14	F: GAGGGTTCACGTTTGTGTC R: GCACCAAGCACAAGAGCAAT	$(GCG)_6$	163	PDK_20s1505351	9121	9138	Probable ascorbate-specific transmembrane electron transporter 1	1.00E-82	*Oryza sativa*
mPdIRD15	F: CCGAGTCTGGCGAAGTAAAC R: CTCCCCTTCCTCATCCTCTC	$(GAA)_6$	406–408	PDK_20s1507261	2378	2395	Eukaryotic translation initiation factor 2 subunit beta	1.00E-22	Wheat
mPdIRD16	F: CTGTCCGATCGAATTCTGC R: GGACATCTCTTTGCGGTCAT	$(CAG)_6$	197–214	PDK_20s1521921	7038	7055	Probable WRKY transcription factor 41	3.00E-47	*Arabidopsis thaliana*
mPdIRD17	F: GTGGAGAAACCCGAAGAAT R: CTGCTGCCTCATCTGCATT	$(AGC)_6$	199–202	PDK_20s1549911	54838	54855	Flowering time control protein FCA	3.00E-38	*Arabidopsis thaliana*
mPdIRD20	F: TTGAATGGTCCCCTGTAGGT R: GTCCCAGCATGATTGCAGTA	$(AGT)_6$	341–373	PDK_20s1640771	6702	6719	Transcription factor bHLH62	7.00E-57	*Arabidopsis thaliana*
mPdIRD22	F: GGCTGTATGGGAAAGACCTG R: CCTGCTGCATATTCTTCGTG	$(GAA)_6$	231–271	PDK_20s1726541	2878	2895	Probable peptide/nitrate transporter At1g59740	4.00E-40	*Arabidopsis thaliana*
mPdIRD24	F: GCTCCTGCAGAACCTGAAAC R: GGACATCACCGTCCAATTCT	$(AAG)_6$	184	PDK_20s1762671	5194	5211	Probable nucleolar protein 5-1	2.00E-46	*Arabidopsis thaliana*
mPdIRD25	F: CACTGGAAATTCAGGGCCTA R: CCCAATTTCTCAGCCAAGAC	$(AGG)_6$	193–205	PDK_20s1831761	4692	4709	Heat stress transcription factor A-2c	8.00E-135	*Oryza sativa*
mPdIRD26	F: CCTCCAGTTCATGCTTCTCC R: GAGCAGACCCGACAGACAAT	$(ACC)_7$	189–192	PDK_20s130094114	13441	13461	Protein transport protein Sec24-like At3g07100	4.00E-99	*Arabidopsis thaliana*
mPdIRD28	F: GAAACGGTATCGGGATGATG R: TTAACGACGCCGTTTCCT	$(TGA)_7$	299–306	PDK_20s1327431	28753	28773	Nuclear cap-binding protein subunit 2	3.00E-82	*Arabidopsis thaliana*

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TABLE 1. Continued.

Locus	Primer sequences (5′–3′)[a]	Repeat motif	Size range (bp)[b]	Scaffold ID	Start	Stop	Gene annotation	*E*-value	Organism
mPdIRD29	F: GGCTCCACCATCATTGACA	$(CCA)_7$	205–217	PDK_20s1359471	804	824	Putative pectinesterase 14	1.00E-34	*Arabidopsis thaliana*
	R: AACAGCATCGACTGCCTTCT								
mPdIRD30	F: GCAGATGGTTGAAAGCTCCT	$(TCA)_7$	218–224	PDK_20s1398581	15353	15373	No hit		
	R: CCCCATTAACAGGATCAACG								
mPdIRD31	F: GCAGGTGGACTGCAAAATCT	$(CCA)_7$	343–372	PDK_20s1419261	29072	29092	Flowering time control protein FY	4.00E-76	*Arabidopsis thaliana*
	R: CTATTGGGGTGCTGATCCAT								
mPdIRD32	F: AAGAAGACATTCCGGCTGGT	$(ATC)_7$	148–163	PDK_20s1457341	3172	3192	Probable alpha-glucosidase Os06g0675700	0.0	*Oryza sativa*
	R: GCGGGTGTGTGATATTGATG								
mPdIRD33	F: GGAGCATACAGTGGGTTTGC	$(CAG)_7$	189–213	PDK_20s1569281	5206	5226	Putative clathrin assembly protein At4g25940	6.00E-133	*Arabidopsis thaliana*
	R: CAGCCTGGGAATGAGGATAG								
mPdIRD35	F: CAGCCCCTTACTCAGACTGG	$(GCA)_7$	209	PDK_20s1690511	5056	5076	No hit		
	R: CCCATAAGCTGATTGTGCTG								
mPdIRD36	F: GACACGTTGACGATGTGGAA	$(TCA)_8$	162–177	PDK_20s1457341	3210	3233	Probable alpha-glucosidase Os06g0675700	0.0	*Oryza sativa*
	R: CCATTGCTGTTGAGGAGGAG								
mPdIRD37	F: TTTCCTGCTCGAAAGACACC	$(AGC)_9$	171–191	PDK_20s1521781	15593	15619	Hydroxyphenylpyruvate reductase	3.00E-71	*Solenostemon scutellarioides*
	R: CTTAGCCAGCCTCCACACTC								
mPdIRD40	F: GAGAGATGCGTCAGGGAATC	$(CCAGTG)_4$	175–211	PDK_20s1327401	16193	16216	No hit		
	R: CCAGAATCTTCCAAGCAAGC								
mPdIRD42	F: GAGGCAAAACTATGGGAAGC	$(CCAGCA)_4$	82–86	PDK_20s1397171	13789	13812	Histone-lysine *N*-methyltransferase SUVR2	6.00E-04	*Arabidopsis thaliana*
	R: TTCACTGGAGCCAAGGGTAGG								
mPdIRD43	F: GCAGCCATTGCTTACAGTGA	$(AACCCT)_4$	202–208	PDK_20s1411101	2862	2885	Chaperone protein ClpB1	2.00E-05	*Arabidopsis thaliana*
	R: TAAACTGCTGCCTTCTTTGG								
mPdIRD44	F: CAGATCCGGGAGATGATGAA	$(TGGTGC)_4$	263	PDK_20s1467201	3121	3144	Two-component response regulator ARR2	2.00E-06	*Arabidopsis thaliana*
	R: AGCAGGAGCAGCTGCATAA								
mPdIRD45	F: TAGCCTGTGCATGTTCGTTG	$(AGCATC)_4$	197	PDK_20s1473281	13788	13811	No hit		
	R: AACAGCAGCTGATGGTGATG								
mPdIRD46	F: ATGGGTCCATTGGAGGAACT	$(CAGGCA)_4$	173–197	PDK_20s1677871	3983	4006	Protein spotted leaf 11	0.0	*Oryza sativa*
	R: GACGGAGACCTTGACTGCTC								

[a] Annealing temperature for all primers is 60°C.
[b] Size ranges were compiled from all amplification experiments conducted on seven *Phoenix* species.

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P. reclinata Jacq. (2), *P. roebelenii* O'Brien (2), *P. rupicola* T. Anderson (2), *P. theophrasti* Greuter (2), and the interspecific hybrid *P. canariensis* × *P. sylvestris* (Table 2). Among these loci, 15 showed consistent amplification and promising polymorphisms across the sample and were further investigated in a variable number of individuals (80–1000) of the aforementioned species, including population samplings of *P. dactylifera* and *P. reclinata*. The transferability of 10 loci was also evaluated in *Chamaerops humilis* L., resulting in 100% positive amplification, with eight polymorphic loci displaying two to 12 alleles among seven to 51 individuals (Table 3). Moreover, the amplification of one *Hyphaene thebaica* Mart. individual and one *Livistona carinensis* (Chiov.) J. Dransf. & N.W. Uhl individual was tested for five loci, with both species giving positive amplification results in three loci (mPdIRD25, mPdIRD31, and mPdIRD33).

DNA from these individuals was extracted from freeze-dried or silica-dried leaf tissue. Samples were reduced into a fine powder using either an IKA A10 analytical grinder (IKA-Werke, Staufen, Germany) or a QIAGEN TissueLyser and QIAGEN DNeasy Plant Mini, Maxi, or 96-well kits (QIAGEN, Courtaboeuf, France). PCR reactions were performed in a thermocycler (Biometra GmbH, Göttingen, Germany, or Eppendorf AG, Hamburg, Germany) in a total reaction mixture of 25 µL, containing: 10 ng of total genomic DNA, 1× PCR buffer, 2 mM $MgCl_2$, 200 µM dNTP, 0.5 U of *Taq* DNA polymerase, 0.4 pmol of the forward primer labeled with a 5′ M13 tail, 2 pmol of the reverse primer, and 2 pmol of the fluorochrome-marked M13 tail, plus sterile water to reach the final volume. The fluorochromes used were either 6-FAM, HEX, or TAMRA. The PCR parameters were as follows: denaturation for 2 min at 94°C; followed by six cycles at 94°C for 45 s, 60°C for 1 min, and 72°C for 1 min; then 30 cycles at 94°C for 45 s, 55°C for 1 min, and 72°C for 1.5 min; then 10 cycles at 94°C for 45 min, 53°C for 1 min, 72°C for 1.5 min; and a final elongation step at 72°C for 10 min.

The PCR products were processed on an ABI 3130XL Genetic Analyzer (Applied Biosystems, Foster City, California, USA). Allele size scoring was performed with respect to a noncommercial ladder using GeneMapper version 3.7 software (Applied Biosystems).

Genetic analyses (number of alleles, observed and expected heterozygosities, Wright's fixation index [F_{IS}] and its significance calculated using the permutation test) were conducted with GENETIX version 4.05 software (Belkhir et al., 2004).

Each of the 15 loci tested were polymorphic in at least one *Phoenix* species (Tables 2 and 3). The loci mPdIRD25, mPdIRD30, mPdIRD31, mPdIRD33, and mPdIRD40 were particularly suitable in *P. dactylifera* with three to eight alleles, having a clear stepwise mutation pattern in accordance with the microsatellite motif (tri- or hexanucleotide), and showing little to moderate heterozygosity deficit. The loci mPdIRD13, mPdIRD25, mPdIRD31, and mPdIRD33 were useful in *Chamaerops humilis* with three to 12 alleles, confirming good intergeneric transferability. In addition, mPdIRD25, mPdIRD31, and mPdIRD33 were amplified in *Livistona carinensis* and *Hyphaene thebaica*.

Table 2. Test of functionality of the 33 loci across the *Phoenix* genus.[a]

Locus	Pdac (7)	Prec (2)	Proe (2)	Prup (2)	Pthe (2)	Phyb (1)	All (16)	SM[b]	Locus comment
mPdIRD01	M	M	M	M	M	M	M	—	100% amplification, monomorphic
mPdIRD03	P	M	M	M	Failed	Failed	P	3	Partial amplification, intra- or interspecific polymorphism
mPdIRD04	M	M	M	M	M	M	P	3	100% amplification, interspecific polymorphism
mPdIRD05	M	M	M	M	M	M	P	No	100% amplification, interspecific polymorphism
mPdIRD07	M	M	M	M	P	M	P	3	Partial amplification, intra- or interspecific polymorphism
mPdIRD08	M	Failed	Failed	Failed	Failed	Failed	M	—	Partial amplification, monomorphic
mPdIRD10	P	P	M	Failed	M	Failed	P	3	Partial amplification, intra- or interspecific polymorphism
mPdIRD11	P	P	P	M	M	M	P	3	100% amplification, intra- or interspecific polymorphism
mPdIRD13	P	P	P	M	P	M	P	3	100% amplification, intra- or interspecific polymorphism
mPdIRD14	M	Failed	Failed	Failed	Failed	Failed	M	—	Partial amplification, monomorphic
mPdIRD15	M	M	M	M	M	P	P	No	100% amplification, interspecific polymorphism
mPdIRD16	P	M	M	M	M	M	P	3	100% amplification, intra- or interspecific polymorphism
mPdIRD17	M	M	M	M	M	P	P	3	100% amplification, interspecific polymorphism
mPdIRD20	M	P	M	M	P	M	P	3	100% amplification, intra- or interspecific polymorphism
mPdIRD22	M	M	M	P	M	M	P	3	100% amplification, intra- or interspecific polymorphism
mPdIRD24	M	M	M	M	M	M	M	—	100% amplification, monomorphic
mPdIRD25	P	P	M	M	M	M	P	3	100% amplification, intra- or interspecific polymorphism
mPdIRD26	P	M	M	M	M	M	P	3	Partial amplification, intra- or interspecific polymorphism
mPdIRD28	P	M	P	M	M	M	P	No	100% amplification, intra- or interspecific polymorphism
mPdIRD29	P	P	M	Failed	Failed	Failed	P	3	Partial amplification, intra- or interspecific polymorphism
mPdIRD30	P	P	Failed	Failed	M	Failed	P	3	Partial amplification, intra- or interspecific polymorphism
mPdIRD31	P	P	M	M	M	M	P	3	100% amplification, intra- or interspecific polymorphism
mPdIRD32	M	P	M	M	M	P	P	3	100% amplification, intra- or interspecific polymorphism
mPdIRD33	P	P	M	M	M	M	P	3	100% amplification, intra- or interspecific polymorphism
mPdIRD35	M	M	M	M	M	M	M	—	100% amplification, monomorphic
mPdIRD36	M	P	M	M	M	P	P	3	100% amplification, intra- or interspecific polymorphism
mPdIRD37	P	P	P	P	M	P	P	3	Partial amplification, intra- or interspecific polymorphism
mPdIRD40	P	P	M	M	P	P	P	3	100% amplification, intra- or interspecific polymorphism
mPdIRD42	P	Failed	Failed	Failed	Failed	Failed	P	No	Partial amplification, intra- or interspecific polymorphism
mPdIRD43	P	M	M	M	M	M	P	6	100% amplification, intra- or interspecific polymorphism
mPdIRD44	P	Failed	Failed	Failed	Failed	Failed	P	No	Partial amplification, intra- or interspecific polymorphism
mPdIRD45	M	Failed	Failed	Failed	Failed	Failed	M	—	Partial amplification, monomorphic
mPdIRD46	P	P	P	P	P	P	P	6	100% amplification, intra- and interspecific polymorphism

Note: M = monomorphic; P = polymorphic; Pdac = *Phoenix dactylifera*; Prec = *Phoenix reclinata*; Proe = *Phoenix roebelenii*; Prup = *Phoenix rupicola*; Pthe = *Phoenix theophrasti*; Phyb = *Phoenix canariensis* × *Phoenix sylvestris*; SM = stepwise mutation pattern.

[a] Species abbreviations are presented with the number of samples tested in parentheses. Herbarium voucher information: Pdac = *dac1*: cultivated, Kew, United Kingdom, MWC 1395 (K); *dac2*: cultivated, Elche, Spain, cv. 'Zahidi', MWC 1800/Barrow 77 (K); *dac3*: cultivated, Kew, MWC 1891 (K); *dac4*: cultivated, Kew, MWC 1398/Kew 1987-3379 (K); *dac5*: cultivated, Kew, MWC 1164 (K); *dac6*: feral, Gran Canaria, Pintaud 636 (G); *dac7*: cultivated Faisalabad, Pakistan, cv. 'Khadrawy', Pintaud 648 (G); Prec = *rec1*: Djibouti, Pintaud 642 (G); *rec2*: Zimbabwe, MWC 1874/Wilkin 724 (K); Proe = *roe1*: cultivated, Thailand, MWC 1161/Barrow 26 (K); *roe2*: cultivated, United Kingdom, MWC 1400/Kew 1987-530; Prup = *rup1*: cultivated, United Kingdom (from India), Pintaud 586 (G); *rup2*: Samchi, Bhutan, MWC 1162/Grierson and Long 3414 (K); Pthe = *the1*: cultivated, Sanremo, Italy, Pintaud 646 (G); Phyb = cultivated, Sanremo, Italy, no. 91005.

[b] In cases where stepwise mutation occurs, the number of base pairs of the repeat unit is given.

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TABLE 3. Polymorphism characterization for 15 loci in *Phoenix* and 10 loci in *Chamaerops*.

	Phoenix all/Pdac/Prec		*Phoenix dactylifera*			*Chamaerops humilis*	
Locus	*N*	*A*	H_o	H_e	F_{IS}	*N*	*A*
mPdIRD11	18/9/2	2/2/2	—	—	—	—	—
mPdIRD13	700/560/25	10/2/4	—	—	—	7	4
mPdIRD16	100/87/2	3/2/1	—	—	—	7	2
mPdIRD20	100/87/2	5/1/2	—	—	—	7	5
mPdIRD22	100/87/2	5/1/1	—	—	—	—	—
mPdIRD25	300/108/60	5/4/2	0.29	0.42	0.31*	51	3
mPdIRD28	184/108/15	9/4/3	0.06	0.44	0.85*	—	—
mPdIRD30	83/28/15	4/3/2	0.11	0.10	–0.04	—	—
mPdIRD31	850/573/85	12/4/4	0.19	0.20	0.03	51	3
mPdIRD32	186/108/15	6/1/4	—	—	—	51	2
mPdIRD33	1000/618/85	12/4/8	0.19	0.23	0.16*	51	12
mPdIRD36	186/108/15	5/1/3	—	—	—	51	1
mPdIRD40	1000/645/85	11/8/6	0.47	0.53	0.11*	51	2
mPdIRD43	100/87/2	2/2/1	—	—	—	7	1
mPdIRD46	80/32/5	6/3/3	—	—	—	—	—

Note: *A* = number of alleles; F_{IS} = fixation index for inbreeding within populations; H_e = expected heterozygosity; H_o = observed heterozygosity; *N* = number of individuals tested; Pdac = *Phoenix dactylifera*; *Phoenix* all = all individuals of seven *Phoenix* species; Prec = *Phoenix reclinata*.

* Significant departure from Hardy–Weinberg equilibrium.

CONCLUSIONS

The loci described here are a useful addition to previously published microsatellite markers for palms. Their interspecific allelic differentiation makes them particularly suitable for hybrid and gene flow analysis within *Phoenix*. The most polymorphic loci can be added to other SSR loci to create marker sets for genetic diversity analysis in *P. dactylifera* and other species. Their transferability within the Coryphoideae subfamily will facilitate the study of species with limited molecular resources, such as *Chamaerops humilis*.

LITERATURE CITED

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