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TECHNICAL NOTE

Development of microsatellite markers for the northern Australian endemic fan palm *Livistona rigida* (Arecaceae), with cross-amplification in the five related species

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Abstract We developed and characterized nine microsatellite markers for *Livistona rigida,* a species endemic to northern Australia, and confirmed the transferability of these markers for five other Australian *Livistona* species. The number of alleles per locus ranged from 1 to 6 with an average of 2.1, and the expected heterozygosity ranged from 0.00 to 0.73 with averages of 0.23. Most of the loci were successfully amplified and showed moderate to high polymorphism for five other *Livistona* species sampled. The microsatellite markers described here will be useful for investigating the speciation and range formation processes of the recent radiation of the *Livistona* genus in Australia, and the results obtained from them will provide crucial information for conservation of Australian *Livistona* species.

Keywords Fan palm · *Livistona mariae* · *L. rigida* · Speciation · SSR marker

Eighteen of the 36 species of the fan palm genus *Livistona* are restricted to Australia. *Livistona rigida* Beccari (Arecaceae) is an endemic palm which mainly occurs in two disjunct regions of northern Australia (Dowe 2009; Rodd 1998). These populations are separated by about 1,000 km from their nearest congener *L. mariae* which is endemic to the Palm Valley Oasis in arid central Australia. Although these two species show significant geographical isolation both between species and among populations, they are identical across 4 kb of chloroplast and nuclear sequences (Crisp et al. 2010). Therefore, the origin and the process of species range formation of these two species are uncertain (Byrne et al. 2008; Crisp et al. 2010). Population-level analyses based on microsatellite polymorphism will help to reveal the speciation and range formation processes of *L.rigida* and *L. mariae*, as well as other closely related *Livistona* palms in Australia. In addition, microsatellites will provide crucial information for conservation of *Livistona* species in Australia, some of which are extremely restricted in geographic range. We developed nine microsatellite markers for *L. rigida*, and investigated the transferability of these markers in the closely related *L. mariae* and four less closely related Australian *Livistona* species (Crisp et al. 2010).

Microsatellite markers were developed using the improved technique for isolating codominant compound microsatellite markers of Lian and Hogetsu (2002) and Lian et al. (2006). An adaptor-ligated, restricted DNA library for *Lvistona rigida* was constructed according to the following procedure: genomic DNA was extracted from fresh leaves using a modified CTAB method (Milligan 1992) and digested with the blunt-end restriction enzyme *Eco*RV. The restriction fragments were then ligated with a specific blunt adaptor (consisting of the 48-mer: 5′-GTAA TACGACTCACTATAGGGCACGCGTGGTCGACGGCC CGGGCTGGT-3′ and an 8-mer with the 3′-end capped with an amino residue: 5′-ACCAGCCC-NH_2-3′) using the

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Takara DNA ligation kit. Fragments were amplified by PCR from the *Eco*RV DNA library using compound SSR primer $(AC)_6(AG)_5$ or $(TC)_6(AC)_5$ and an adaptor primer (5′-CTATAGGGCACGCGTGGT-3′). The amplified fragments were cloned using the QIAGEN PCR Cloning Kit (Qiagen) following the manufacturer's instructions. The cloned fragments were amplified using the M13 forward and reverse primers from the plasmid DNA. Amplified fragments were sequenced using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems). For each fragment containing a compound SSR sequence at one end, a specific primer was designed from the sequence flanking the compound SSR using Primer3 (v. 0.4.0, Rozen and Skaletsky 2000) (National Human Genome Research Institute). Polymerase chain reaction amplifications were performed following the standard protocol of the Qiagen Multiplex PCR Kit (Qiagen) in a final volume of 10 µl, which contained 5 ng of extracted DNA, 5 µl of 2× Multiplex PCR Master Mix, and 0.2 µM of each multiplexed primer. Compound SSR primers ($(AC)_6(AG)_5$ or $(TC)_6(AC)_5$) were labeled with fluorochromes 6-FAM or VIC (Applied Biosystems), respectively. Polymerase chain reaction amplifications were performed with the GeneAmp PCR System 2700 thermal cycler (Applied Biosystems) using the following conditions: initial denaturation at 95°C for 15 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing for each of the designed specific primers at the temperatures shown in Table 1 for 1 min 30 s, extension at 72°C for 1 min, and final extension at 60°C for 30 min. The size of the PCR products was measured using the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems) and GENOTYPER software (Applied Biosystems).

Nine loci were identified that showed a clear, strong single band for each allele (Table 1). The polymorphism was evaluated for 30 individuals from the three populations (Roper River, Nicolson-Gregory catchment 1 and 2) of *L. rigida* in Northern Australia. Deviation from Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium between loci were tested using FSTAT (version 2.9.3; Goudet 1995). Significance levels were adjusted using Bonferroni correction for multiple testing. At population level, the number of alleles per locus (A) ranged from 1 to 6 with an average of 2.1, and the observed and expected heterozygosity (H_O and H_E) ranged from 0.00 to 1.00 and from 0.00 to 0.73, with averages of 0.27 and 0.23, respectively. No

Table 1 Characteristics of nine compound microsatellite loci for *Livistona rigida*

Locus	Repeat motif	Primer sequences (5–3′)	T_a (°C)	Size range (bp)	Accession No.	Roper river ($n = 10$)			Nicholson-Gregory catchment 1 ($n = 10$)			Nicholson-Gregory catchment 2 ($n = 10$)		
						A	H_O	H_E	A	H_O	H_E	A	H_O	H_E
LR03	$(AC)_6(AG)_{17}$	ACACACACACACAGAGAGAGAG CATAGGAAGGTTTTGACAG	57	178–200	AB621622	6	1.00	0.73	2	0.30	0.26	2	0.10	0.10
LR10	$(AC)_6(AG)_5$	ACACACACACACAGAGAGAGAG CCAGGATGTCACTATTTAAG	57	112	AB621623	1	0.00	0.00	1	0.00	0.00	1	0.00	0.00
LR13	$(AC)_6(AG)_{31}$	ACACACACACACAGAGAGAGAG GTGATGGTGGTAGACTTATT	57	97–123	AB621624	4	0.70	0.69	2	0.50	0.38	4	0.90	0.59
LR14	$(AC)_6(AG)_{21}$	ACACACACACACAGAGAGAGAG GGTGAGAGAGTGAATGAGAA	57	92–104	AB621625	3	0.70	0.49	4	0.30	0.58	4	0.60	0.70
LR22	$(AC)_6(AG)_{17}$	ACACACACACACAGAGAGAGAG CTTGTCACCATCATCATCAC	57	104–126	AB621626	2	0.10	0.10	1	0.00	0.00	1	0.00	0.00
LR23	$(AC)_6(AG)_{25}$	AGAGAGAGAGAGACACACACAC GAACTGATGAGCTTTTTATA	57	100–104	AB621627	2	0.10	0.10	2	0.10	0.10	2	0.10	0.10
LR24	$(AC)_6(AG)_{12}$	AGAGAGAGAGAGACACACACAC AACTAAACTGCAAAACCTA	57	100–104	AB621628	1	0.00	0.00	2	0.20	0.18	1	0.00	0.00
LR38	$(TC)_6(AC)_6$	TCTCTCTCTCTCACACACACAC GCGAAGAGATCTAATAGAA	57	50–60	AB621629	1	0.00	0.00	1	0.00	0.00	1	0.00	0.00
LR46	$(TC)_6(AC)_{16}$	TCTCTCTCTCTCACACACACAC ACATCCAGGAAAGAATATA	57	140–148	AB621630	2	0.20	0.18	2	0.60	0.48	3	0.70	0.54
					Average	2.4	0.31	0.25	1.9	0.22	0.22	2.1	0.27	0.22

T_a annealing temperature of primer pair

Table 2 Variability of nine microsatellite loci for five *Livistona* species in Australia

	L. mariae (n = 20)			*L. humilis* (n = 10)			*L. inermis* (n = 10)			*L. lanuginosa* (n = 20)			*L. nasmophila* (n = 18)		
	A	H_O	H_E	A	H_O	H_E	A	H_O	H_E	A	H_O	H_E	A	H_O	H_E
LR03	3	0.15	0.56	7	0.80	0.79	8	0.60	0.81	7	0.70	0.68	2	0.17	0.15
LR10	6	0.80	0.60	2	0.10	0.10	1	0.00	0.00	1	0.00	0.00	2	0.06	0.05
LR13	1	0.00	0.00	8	0.80	0.84	11	1.00	0.88	3	0.15	0.43	2	0.06	0.15
LR14	2	0.05	0.05	10	0.80	0.84	8	0.80	0.76	5	0.70	0.61	4	0.39	0.48
LR22	2	0.05	0.05	11	0.80	0.89	7	0.70	0.80	1	0.00	0.00	3	0.22	0.20
LR23	2	0.20	0.18	10	0.90	0.85	5	0.30	0.42	3	0.40	0.54	6	0.72	0.59
LR24	1	0.00	0.00	4	0.40	0.58	7	0.80	0.69	2	0.50	0.50	3	0.50	0.53
LR38	1	0.00	0.00	8	0.40	0.84	4	0.40	0.62	1	0.00	0.00	X	–	–
LR46	2	0.00	0.10	X	–	–	0	–	–	2	0.40	0.46	3	0.17	0.16
Average	2.2	0.14	0.17	7.5	0.63	0.71	5.7	0.58	0.62	2.8	0.32	0.36	3.1	0.28	0.29

X indicate that the locus was not amplified

n number of individuals genotyped, A number of alleles per locus, H_O observed heterozygosity, H_E expected heterozygosity

significant deviations from HWE ($P < 0.05$) were observed at any loci, and there was no evidence of significant linkage disequilibrium ($P < 0.05$) for all pair of loci.

All nine loci were screened in cross-amplification tests for five other Australian *Livistona* species, *L. mariae, L. humilis, L.inermis, L. lanuginosa*, and *L. nasmophila*. In the cross amplification of the five *Livistona* species, most of nine loci were successfully amplified and showed moderate to high polymorphism (Table 2). No significant deviations from HWE and no significant linkage disequilibrium were detected. The microsatellite markers described here will be useful for investigating the speciation and range formation processes of the recent radiation of *Livistona* palms in Australia, and the results obtained from them will provide crucial information for conservation of *Livistona* species in Australia.

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