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TECHNICAL NOTE

A set of polymorphic nuclear intron markers for conservation genetics and phylogeography of *Euphorbia* species (*Pedilanthus* clade)

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Abstract We developed seven nuclear intron markers for *Euphorbia lomelii*. New exon-primed intron-crossing (EPIC) oligonucleotides were used for initial amplification and sequencing, then locus-specific primers and restriction-fragment-length polymorphism genotyping assays were designed. Loci showed no significant deviation from Hardy–Weinberg and linkage equilibrium, and they cross-amplify in at least three congeneric species.

Keywords Co-dominant loci · Euphorbiaceae · Population structure · Phylogeny · Sonoran Desert

Euphorbia is an extremely diverse plant genus, with over 2,000 species worldwide. The *Pedilanthus* clade is of immediate conservation concern—several members are threatened or endangered, and most species tolerate only relatively little disturbance (Olson et al. 2005). *Euphorbia lomelii* (synon. *Pedilanthus macrocarpus*) is endemic to the Baja California peninsula and a small adjacent section of mainland Mexico, separated by the Sea of Cortez. The species displays considerable diversity in growth form, and based on significant among-population variation in hydrocarbon chemistry, it is hypothesized to comprise at least two races (Sternburg and Rodriguez 1982). To date, no population-genetic studies have been conducted for any member of the *Pedilanthus* clade, yet such information is critical for effective conservation. Here we report the first set of polymorphic nuclear markers for *Euphorbia lomelii*, and demonstrate cross-species transferability in three other clade members. Analysis of DNA sequence variation indicated that these introns carry considerable phylogenetic signal, extending their utility to phylogeographic applications.

GenBank was surveyed for accessions of selected low-copy nuclear genes (most searches constrained within the Rosids), then downloaded sequences were aligned and inspected for introns, as indicated by alignment gaps causing frameshifts when translated. Degenerate EPIC primers were designed in flanking exons of the following genes: granule-bound starch synthase (*Wxy*), floral meristem identity protein (*Lfy*), alcohol dehydrogenase (*Adh*), pistillata (*Pi*), RNA polymerase II (*Rpb2*), and glyceraldehyde 3-phosphate dehydrogenase (*G3pdh*) (Supplementary Material Table S1, Fig. S1).

Euphorbia lomelii genomic DNA was extracted using a DNeasy® plant tissue kit (Qiagen). Polymerase chain reactions (PCRs) were performed using a PTC-200 thermocycler (MJ Research) in 10 μl volumes containing 2 μl 5× *GoTaq*® Flexi buffer (Promega), 2 mM $MgCl_2$ 200 μM each dNTP, 5% bovine serum albumin (BSA) (New England Biolabs, NEB), 0.5 μM each primer, 0.5 U *GoTaq*® DNA polymerase (Promega), and 1 μl template DNA. EPIC primers were tested on 8–10 individuals, using the following profile: 95°C 2 min (1 cycle), 95°C 30 s, 44°C or 48°C 30 s (i.e. two reactions per sample), 72°C 1 min (35 cycles), 72°C 2 min (1 cycle). Amplification products were electrophoresed through 2% agarose gels stained with ethidium bromide, viewed using ultraviolet light. Discrete bands of similar sizes

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Table 1 Characterization of nuclear intron loci developed for *Euphorbia lomelii*

Locus name	[a] Primer sequence (5′-3′)	T (°C)	Amplicon sizes (bp)	RFLP assay	N	Population 1			Population 2			GenBank accessions
						H_O	H_E	P-value	H_O	H_E	P-value	
EI*Wxy*	F: TATAAGGACGCATGGGATAC R: AAGAGATGAGAGCGATAGGGAAG	46	244	*BsaB I*	2	0.516	0.486	0.697	Mono	–	–	EU287540–EU287541
EI*Lfy*	F: ACGTGTCAAAAAGGTACGGACTGG R: TGATAGAGGTGCAGAACATTGC	46	325	*Hsp92 II*	2	0.097	0.124	1.000	0.200	0.220	1.000	EU287506–EU287507
EI*Adh*	F: AGAATGCAGGGAATGTGCTC R: ACTGTGTACTCACTGAAAGTGG	50	167, 171	*BtsC I*	2	0.194	0.207	1.000	Mono	–	–	EU287489–EU287490
EI*Pi*	F: AAGAGTCCTCAGCATACTTC R: TCTGGTGCCTTCTGAGGTAC	50	463	*Afi II*	3	0.355	0.500	0.068	Mono	–	–	EU287522–EU287524
EI*Rpb2*	F: TGGAGACAAATTCAGTAGTC R: AATAACCGAAGTTCCTACCTCG	50	288, 293	*Nde I*	3	0.323	0.442	0.218	0.160	0.089	1.000	EU287533–EU287537
EI*Ms*	F: TCATGGTGATTACAGCTCAATCC R: TGGAAAGCAGCATAATTATGGTAG	46	355, 356	*Rsa I*	2	Mono	–	–	0.120	0.153	1.000	EU287512–EU287515
EI*G3pdh*	F: TTCATCTTTCCCTCAGACTCC R: AGCTGCCAAGGTAGAAATTGCACGC	50	358	Not available	1	Mono	–	–	Mono	–	–	EU287497–EU287499

[a] See Supplementary Material for sequence of EPIC primers designed from GenBank accessions for initial amplification of targeted nuclear introns

T, annealing temperature; RFLP, restriction fragment length polymorphism; N, number of alleles resolved; H_O, observed heterozygosity; H_E, expected heterozygosity; Mono, monomorphic

Table 2 Cross-species amplification of nuclear intron loci using *Euphorbia lomelii* primers

Locus name	Species	*T* (°C)	Amplicon sizes (bp)	No. of loci amplified	[a] Polymorphism (No. sequenced)	GenBank accessions
EIW*xy*	*E. tithymaloides*	46	245	1	✘ (2)	EU287542
	E. calcarata	46	245	1	✘ (2)	EU287543
	E. personata	46	245	1	✘ (2)	EU287544
EIL*fy*	*E. tithymaloides*	46	325, 326	1	✔ (2)	EU287508–EU287509
	E. calcarata	46	325	1	✘ (2)	EU287510
	E. personata	46	325	1	✘ (2)	EU287511
EIA*dh*	*E. tithymaloides*	46	171	2	? (1)	EU287491, EU287494
	E. calcarata	46	166, 171	2	? (1)	EU287492, EU287495
	E. personata	46	171	2	? (1)	EU287493, EU287496
EI*Pi*	*E. tithymaloides*	50	463	1	✔ (2)	EU287525–EU287526
	E. calcarata	50	437, 463	1	✔ (4)	EU287527–EU287530
	E. personata	50	463	1	✔ (2)	EU287531–EU287532
EI*Rpb2*	*E. tithymaloides*	52	293	1	? (1)	EU287538
	E. calcarata	46	293	1	✘ (2)	EU287539
	E. personata	46	293	?	? (0)	
EI*Ms*	*E. tithymaloides*	46	355	1	✔ (2)	EU287516–EU287517
	E. calcarata	46	355	1	✔ (2)	EU287518–EU287519
	E. personata	46	355	1	✔ (3)	EU287520–EU287521
EIG3*pdh*	*E. tithymaloides*	46	358	1	✔ (2)	EU287500–EU287501
	E. calcarata	46	358	1	✔ (3)	EU287502–EU287504
	E. personata	46	358	1	? (1)	EU287505

[a] Intraspecific DNA sequence variation confirmed (✔), not confirmed (✘), or unknown(?)

T, annealing temperature; No. of loci amplified, co-amplification of putative paralogs inferred by sequencing; No. sequenced, number of amplicons selected following double-stranded conformation polymorphism assays using 6% non-denaturing acrylamide gels run as 50 V 10 mA for 270 min, at 4°C

(± 50-bp) were excised and eluted in 15 μl dH_2O overnight, then used as template for reamplification (reaction mix and profile as above, but with 55°C annealing). Sequencing of diploid PCR products was performed on a MegaBACE 1000 (Amersham Biosciences). Using the same procedure, we also trialed malate synthase (*Ms*) primers 'ms400f' and 'ms943r' (Lewis and Doyle 2001). Non-degenerate species-specific primers for each locus were designed from multi-allele alignments, and annealing temperatures were optimized empirically (Table 1). These primers were tested on five individuals of *E. tithymaloides*, *E. calcarata* and *E. personata* (Table 2). Intraspecific polymorphism in congeners was assessed using double-stranded conformation polymorphism (Saad et al. 1994), followed by sequencing selected amplicons.

Restriction-fragment-length polymorphism (RFLP) assays for resolving known *Euphorbia lomelii* alleles were developed using NEBCUTTER v2.0 (Vincze et al. 2003). Restriction digests were performed in 25 μl volumes containing 2.5 μl recommended 10× buffer (NEB or Promega), 0.25 μl BSA 10 mg/ml (NEB), 2.5 U restriction enzyme (Table 1) and 7 μl PCR product. Reactions were incubated (16 h at recommended temperature) and digestion products separated via electrophoresis (as above). Fragment sizes were estimated using undigested PCR product or a 100-bp ladder (Promega).

One hundred and forty *Euphorbia lomelii* samples were genotyped via PCR-RFLP. Exact tests of Hardy–Weinberg and linkage equilibrium (with Bonferroni correction) were performed on samples from each of two populations ($n = 31$ and $n = 25$) using ARLEQUIN v3.0 (Excoffier et al. 2005). No significant deviation was seen at any of the six loci amenable to analysis (Table 1). All primers amplified homologous introns in three close relatives, and intraspecific polymorphism in at least some congeners was confirmed for four loci (Table 2). Comparative analysis of potentially informative characters per locus, based on alignment of sequences pooled for the four *Euphorbia* species, indicated that pistillata carried the greatest phylogenetic signal relative to other introns (Supplementary Material Fig. S2).

Nuclear introns represent a largely untapped source of genetic variation for population genetics and phylogeography. The polymorphic loci reported here will be used to

investigate the relative influence of landscape history and plant–insect coevolutionary relationships in Sonoran Desert biota.

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